SHOUT
SHARE
IT

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
JAMES CASE, University of California, Santa Barbara
JOHN W. GOWEN, Iowa State College
SALLY HUGHES-SCHRADER, Duke University
LIBBIE H. HYMAN, American Museum of Natural History
SHINYA INOUÉ, Dartmouth College
J. LOGAN IRVIN, University of North Carolina
L. H. KLEINHOLZ, Reed College
JOHN H. LOCHHEAD, University of Vermont
ROBERTS RUGH, Columbia University
WM. RANDOLPH TAYLOR, University of Michigan
CARROLL M. WILLIAMS, Harvard University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

VOLUME 126
FEBRUARY TO JUNE, 1964

MARINE BIOLO
LIBR
MASS.

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $3.75. Subscription per volume (three issues), $9.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P.O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1. February, 1964

Page

Brookbank, John W.
The effect of antisera against fertilization on the uptake of orthophosphate by sea urchin eggs 1

Chaet, Alfred B.
A mechanism for obtaining mature gametes from starfish 8

DeCoursey, George, and Patricia J. DeCoursey
Adaptive aspects of activity rhythms in bats 14

Dinamani, P.
Burrowing behaviour of Dentalium 28

Ferguson, John Carruthers
Nutrient transport in starfish. I. Properties of the coelomic fluid 33

Gross, Warren J.
Water balance in anomuran land crabs on a dry atoll 54

Griffiths, W. M., and A. H. Whiteley
A study of the mechanism of phosphate transport in sea urchin eggs by ion exchange analysis of rapidly labeled compounds 69

Hartman, F. A., and M. A. Lessler
Erythrocyte measurements in fishes, Amphibia, and reptiles 83

Kaan, Helen W.
Development of the amphibian ear rudiment in explants 89

Kallman, Klaus D., and Robert W. Harrington, Jr.
Evidence for the existence of homozygous clones in the self-fertilizing hermaphroditic teleost Rivulus marmoratus (Poey) 101

Lenhoff, Howard M.
Reversible inhibition of swimming in Stomotoca atra by mesogleal extracts of some other medusae 115

Park, Helen D., Christyna E. Mecca and Anne B. Ortmeyer
Carbon dioxide tension and sexual differentiation in Hydra 121

Pax, Ralph A., and Richard C. Sanborn
Cardioregulation in Limulus. I. Physiology of inhibitor nerves 133

Smith, Ralph I.
D_2O uptake rate in two brackish-water nereid polychaetes 142

Stephens, Grover C.
Uptake of organic material by aquatic invertebrates. III. Uptake of glycine by brackish-water annelids 150

Stunkard, Horace W.
The morphology, life-history, and systematics of the digenetic trematode, Homalometron pallidum Stafford, 1904 163

63485

No. 2. April, 1964

Beck, Stanley D., and Nancy Alexander
Chemically and photoperiodically induced diapause development in the European corn borer, Ostrinia nubilalis.......................... 175

Beck, Stanley D., and Nancy Alexander
Proctodone, an insect developmental hormone....................... 185

Black, Robert E., and E. T. Pengelley
Alpha amylase development in embryos of Crassostrea virginica...... 199

Bowers, Blair, and Carroll M. Williams
Physiology of insect diapause. XIII. DNA synthesis during the metamorphosis of the Cecropia silkworm............................. 205

Fisher, Frank M., Jr.
The properties and specificity of a β-glucosidase from Blaberus craniifer. 220

Fisher, Frank M., Jr., and Richard C. Sanborn
Nosema as a source of juvenile hormone in parasitized insects........ 235

Friberg, Ulf A., Philip T. Levine and Melvin J. Glimcher
An air-abrasive method for the microdissection of mineralized tissues.. 253

Hadler, Nortin M.
Genetic influence on phototaxis in Drosophila melanogaster.......... 264

Hochachka, Peter W., and John M. Teal
Respiratory metabolism in a marine dinoflagellate................... 274

Hunn, J. B., and P. O. Fromm
Uptake, turnover and excretion of I-131 by rainbow trout (Salmo gairdneri).. 282

Musacchia, X. J., S. S. Neff and D. D. Westhoff
Active transport of D-glucose by intestinal segments, in vitro, of Ictalurus nebulosus.. 291

Sloan, William C.
The accumulation of nitrogenous compounds in terrestrial and aquatic eggs of prosobranch snails.................................... 302

Spiegel, Melvin, and Evelyn Sclufer Spiegel
The regulation of liver tryptophan pyrrolase activity during the development of Rana catesbiana.................................. 307

Stiven, Alan E.
The effect of the pathogenic rhizopod Hydramoeba hydroxena (Entz) on reproduction in Chlorohydra viridissima under various levels of temperature.. 319

No. 3. June, 1964

Bowen, Sarane Thompson
The genetics of Artemia salina. IV. Hybridization of wild populations with mutant stocks.. 333

Coward, S. J., R. A. Flickinger and E. Garen
The effect of nutrient media upon head frequency in regenerating planaria.. 345

DEHNEL, PAUL A., AND DMITRY STONE
Osmoregulatory role of the antennary gland in two species of estuarine crabs 354
DUNHAM, PHILIP B.
The adaptation of Tetrahymena to a high NaCl environment 373
FERGUSON, JOHN CARRUTHERS
Nutrient transport in starfish. II. Uptake of nutrients by isolated organs 391
GREEN, JONATHAN P.
Morphological color change in the Hawaiian ghost crab, Ocypode ceratophthalma (Pallas) 407
KOBAYASHI, SHINJIRO
Studies on shell formation. X. A study of the proteins of the extrapallial fluid in some molluscan species 414
LOOSANOFF, VICTOR L.
Variations in time and intensity of setting of the starfish, Asterias forbesi, in Long Island Sound during a twenty-five-year period 423
MOHRI, H.
Utilization of C^{14}-labeled acetate and glycerol for lipid synthesis during the early development of sea urchin embryos 440
MONROY, ALBERTO, AND HELEN TOLIS
Uptake of radioactive glucose and amino acids and their utilization for incorporation into proteins during maturation and fertilization of the eggs of Asterias forbesii and Spisula solidissima 456
STUNKARD, HORACE W.
Studies on the trematode genus Renicola: observations on the life-history, specificity, and systematic position 467

Vol. 126, No. 1 February 1964

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE EFFECT OF ANTISERA AGAINST FERTILIZIN ON THE UPTAKE OF ORTHOPHOSPHATE BY SEA URCHIN EGGS[1]

JOHN W. BROOKBANK[2]

Department of Microbiology, College of Medicine, and the Department of Biology, University of Florida, Gainesville, Florida

Antisera against fertilizin have been shown to inhibit the cleavage of the zygote (Tyler and Brookbank, 1956a, 1956b) and to increase the respiratory rate of unfertilized and fertilized eggs of some species of sea urchins (Brookbank, 1959). It has been claimed that antisera against egg homogenates or purified fertilizin are parthenogenic (Perlmann, 1957, 1959). These observations have been re-examined and discussed by Brookbank (1959), Tyler (1959), Tyler *et al.* (1961) and Tyler (1963). No clear evidence of egg activation in the presence of such antisera was found by these authors.

The present report deals with the effect on phosphate uptake of antisera against fertilizin, a process known to increase markedly after fertilization and artificial parthenogenesis (see Whiteley and Chambers, 1960; Litchfield and Whiteley, 1959, for data and earlier references). Phosphate uptake by unfertilized eggs is essentially zero. Further, the transport mechanism is presumed to differentiate at the surface of the egg following activation (Whiteley and Chambers, 1960), and therefore should be available for possible interaction with antibody.

MATERIALS AND METHODS

Eggs and sperm of *Arbacia punctulata* and *Lytechinus variegatus* were obtained by electric shock (Harvey, 1956) or by KCl injection (Palmer, 1937; Tyler, 1949), respectively. Fertilization membranes of *Arbacia* eggs were eliminated by trypsin (1 mg.% for 10 minutes) treatment of unfertilized eggs. Passage of fertilized (1–2 minutes) *Lytechinus* eggs through an 18-gauge needle fixed to a 50-ml. syringe removed the fertilization membranes from most (90%) of these eggs. Fertilizability of the eggs in dilute sperm suspensions was tested after four washes

---

[1] This research was supported in part by a grant (GM 04659) from the National Institutes of Health.

[2] Author's current address: Department of Biology, University of Florida, Gainesville, Florida. The author wishes to gratefully acknowledge the technical assistance of Janet G. Yonge.

Copyright © 1964, by the Marine Biological Laboratory

in sea water, before the eggs were used. Only those of 95–100% fertilizability were used.

Antisera were prepared in rabbits against purified fertilizin, as previously described (Brookbank, 1959). All were sea-water-dialyzed, and all were effective in cleavage inhibition. The antisera against *Lytechinus* fertilizin (4 prime and 10 prime) were the same as those used to determine the effects of such antisera on unfertilized egg respiration (Brookbank, 1959). The antisera against *Arbacia* fertilizin (6 prime and 8 prime) have not yet been tested for respiratory effects. Additional antisera were prepared in rabbits against unfertilized eggs of *Lytechinus* (fertilizin included). Pre-injection sera of all rabbits except 4 and 10 were available for use as controls. Control sera for 4 prime and 10 prime antisera were obtained by bleedings of uninjected animals (abbreviated Th., F., and M_1.).

Phosphate uptake was measured using carrier-free P^{32} (Union Carbide Nuclear Corporation) in sea water. The amount of serum available did not permit the use of the perfusion chamber method of Whiteley and Chambers (1960). In the following experiments, unfertilized or fertilized eggs (0.25 ml. of a 25% or 50% suspension) were placed, in duplicate, in 2-ml. screw-cap vials, together with P^{32}-sea water (50 or 100 mm.^{3}) and serum (0.25 ml.). Sea water controls without serum were included. Fertilized eggs were placed in the vials 30 minutes after fertilization

TABLE I

The effect of normal and immune sera on uptake of P^{32} by fertilized eggs*
*(Figures given are averages of duplicate samples; *, 50% ammonium sulfate fraction; L.v., Lytechinus variagatus; A.p., Arbacia punctulata; I, immune; N, normal; C, sea water control; cpm, counts per minute.)*

Experiment	% Egg concentration	Sera used		Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 mm.^{3} supernatant	Total cpm added to sample
1. *L.v.*	50	4 prime	I	95	63	1815	10,400
		Th.	N		136	1755	
		C			368	1714	
2. *L.v.*	50	4 prime	I	94	92	1771	10,634
		Th.	N		341	1811	
		C			518	1655	
3. *L.v.*	50	4 prime	I	95	506	2306	14,635
		Th.	N		1975	1934	
		C			5895	1338	
4. *L.v.*	50	4 prime	I	100	229	1280	7,317
		Th.	N		570	1227	
		C			3576	704	
5. *L.v.*	25	4 prime	I	89	150	1120	7,201
		Th.	N		472	1136	
		C			1297	936	
6. *L.v.*	25	10 prime	I	92	90	1183	7,320
		F	N		597	1135	
		C			1988	896	

TABLE I—(*Continued*)

Experiment	% Egg concentration	Sera used	Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 mm.^{3} supernatant	Total cpm added to sample
7. *L.v.*	25	4 prime I	95	185	1157	6,938
		M_1 N		266	1195	
		C		3257	566	
8. *L.v.*	25	4 prime I*	91	138	796	4,944
		M_1 N*		148	776	
		C		262	784	
9. *L.v.*	25	a prime I	95	389	1423	9,495
		a N		968	1428	
		C		1198	1282	
10. *A.p.*	25	8 prime I	97	116	1104	12,922
		8 N		263	1436	
		C		652	1442	
11. *A.p.*	25	6 prime I	95	653	1488	9,900
		6 prime (0.45/1) I		1458	1454	
		6 N*		1282	1462	
		C		3360	835	

* Serum code:

4 prime—anti-*Lytechinus* fertilizin
10 prime—anti-*Lytechinus* fertilizin
a prime—anti-*Lytechinus* fertilizin plus unfertilized eggs
6 prime—anti-*Arbacia* fertilizin
8 prime—anti-*Arbacia* fertilizin
6 —corresponding pre-injection sera
8 —corresponding pre-injection sera
a —corresponding pre-injection sera
Th. —normal sera from 3 uninjected rabbits
F. —normal sera from 3 uninjected rabbits
M_1 —normal sera from 3 uninjected rabbits

in order to by-pass the lag and transition phases of phosphate uptake. The length of the lag period varies from female to female within a species (Whiteley and Chambers, 1960). These authors state that an average lag of 15 minutes in P^{32} uptake occurs following fertilization in *Strongylocentrotus purpuratus.* A count of 50 or 100 mm.^{3} of the P^{32}-sea water served to indicate total radioactivity at zero time. After incubating with slow rotation at 1 rpm for 60 minutes at 25° C., the vials were centrifuged and 100 mm.^{3}-aliquots of the supernatant taken. The eggs were washed three times in 2-ml. volumes of cold (0° C.) sea water, quantitatively transferred to planchets, and dried. Some difficulty was experienced in transfer of fertilized, demembranated eggs, in that these eggs sometimes tended to stick to glass. Percentage recovery of radioactivity was calculated after counting the dried samples with a thin-end-window Geiger-Muller tube (1.8 mg./cm.^{2}) within a low background (17 cpm) shield. The results are expressed as counts per minute per 0.25 ml. egg suspension, or per 100 mm.^{3} supernatant sea water. Experiments with 90% recovery or better are included. Comparisons made between experiments are of necessity qualitative since egg suspensions were prepared on the in sea water, before the eggs were used. Only those of 95–100% fertilizability were used.

Antisera were prepared in rabbits against purified fertilizin, as previously described (Brookbank, 1959). All were sea-water-dialyzed, and all were effective in cleavage inhibition. The antisera against *Lytechinus* fertilizin (4 prime and 10 prime) were the same as those used to determine the effects of such antisera on unfertilized egg respiration (Brookbank, 1959). The antisera against *Arbacia* fertilizin (6 prime and 8 prime) have not yet been tested for respiratory effects. Additional antisera were prepared in rabbits against unfertilized eggs of *Lytechinus* (fertilizin included). Pre-injection sera of all rabbits except 4 and 10 were available for use as controls. Control sera for 4 prime and 10 prime antisera were obtained by bleedings of uninjected animals (abbreviated Th., F., and M_1.).

Phosphate uptake was measured using carrier-free P^{32} (Union Carbide Nuclear Corporation) in sea water. The amount of serum available did not permit the use of the perfusion chamber method of Whiteley and Chambers (1960). In the following experiments, unfertilized or fertilized eggs (0.25 ml. of a 25% or 50% suspension) were placed, in duplicate, in 2-ml. screw-cap vials, together with P^{32}-sea water (50 or 100 mm.^{3}) and serum (0.25 ml.). Sea water controls without serum were included. Fertilized eggs were placed in the vials 30 minutes after fertilization

TABLE 1

The effect of normal and immune sera on uptake of P^{32} by fertilized eggs*
*(Figures given are averages of duplicate samples; *, 50% ammonium sulfate fraction; L.v., Lytechinus variagatus; A.p., Arbacia punctulata; I, immune; N, normal; C, sea water control; cpm, counts per minute.)*

Experiment	% Egg concentration	Sera used		Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 mm.^{3} supernatant	Total cpm added to sample
1. *L.v.*	50	4 prime	I	95	63	1815	10,400
		Th.	N		136	1755	
		C			368	1714	
2. *L.v.*	50	4 prime	I	94	92	1771	10,634
		Th.	N		341	1811	
		C			518	1655	
3. *L.v.*	50	4 prime	I	95	506	2306	14,635
		Th.	N		1975	1934	
		C			5895	1338	
4. *L.v.*	50	4 prime	I	100	229	1280	7,317
		Th.	N		570	1227	
		C			3576	704	
5. *L.v.*	25	4 prime	I	89	150	1120	7,201
		Th.	N		472	1136	
		C			1297	936	
6. *L.v.*	25	10 prime	I	92	90	1183	7,320
		F	N		597	1135	
		C			1988	896	

TABLE I—(*Continued*)

Experiment	% Egg concentration	Sera used	Ave. % recovery of P^{32}	Cpm eggs	Cpm 100 mm.^{3} supernatant	Total cpm added to sample
7. *L.v.*	25	4 prime I	95	185	1157	6,938
		M_1 N		266	1195	
		C		3257	566	
8. *L.v.*	25	4 prime I*	91	138	796	4,944
		M_1 N*		148	776	
		C		262	784	
9. *L.v.*	25	a prime I	95	389	1423	9,495
		a N		968	1428	
		C		1198	1282	
10. *A.p.*	25	8 prime I	97	116	1104	12,922
		8 N		263	1436	
		C		652	1442	
11. *A.p.*	25	6 prime I	95	653	1488	9,900
		6 prime (0.45/1) I		1458	1454	
		6 N*		1282	1462	
		C		3360	835	

* Serum code:

4 prime—anti-*Lytechinus* fertilizin
10 prime—anti-*Lytechinus* fertilizin
a prime—anti-*Lytechinus* fertilizin plus unfertilized eggs
6 prime—anti-*Arbacia* fertilizin
8 prime—anti-*Arbacia* fertilizin
6 —corresponding pre-injection sera
8 —corresponding pre-injection sera
a —corresponding pre-injection sera
Th. —normal sera from 3 uninjected rabbits
F. —normal sera from 3 uninjected rabbits
M_1 —normal sera from 3 uninjected rabbits

in order to by-pass the lag and transition phases of phosphate uptake. The length of the lag period varies from female to female within a species (Whiteley and Chambers, 1960). These authors state that an average lag of 15 minutes in P^{32} uptake occurs following fertilization in *Strongylocentrotus purpuratus*. A count of 50 or 100 mm.^{3} of the P^{32}-sea water served to indicate total radioactivity at zero time. After incubating with slow rotation at 1 rpm for 60 minutes at 25° C., the vials were centrifuged and 100 mm.^{3}-aliquots of the supernatant taken. The eggs were washed three times in 2-ml. volumes of cold (0° C.) sea water, quantitatively transferred to planchets, and dried. Some difficulty was experienced in transfer of fertilized, demembranated eggs, in that these eggs sometimes tended to stick to glass. Percentage recovery of radioactivity was calculated after counting the dried samples with a thin-end-window Geiger-Muller tube (1.8 mg./cm.^{2}) within a low background (17 cpm) shield. The results are expressed as counts per minute per 0.25 ml. egg suspension, or per 100 mm.^{3} supernatant sea water. Experiments with 90% recovery or better are included. Comparisons made between experiments are of necessity qualitative since egg suspensions were prepared on the

DISCUSSION

The failure of the antisera to activate the transport mechanism, as do other parthenogenic agents (Whiteley and Chambers, 1960), even though the respiratory rate in the presence of antisera against fertilizin is greatly increased, rules against activation of the eggs by such sera. Most probably, the increased respiration represents an increased oxygen consumption without accompanying oxidative phosphorylation, as with dinitrophenol or other agents (Krahl, 1950). Such uncoupling might be brought about by microscopically invisible cytolytic changes induced by the antisera (Brookbank, 1959).

The inhibition of phosphate uptake of fertilized eggs by antisera and other serum proteins may be ascribed to steric hindrance of transport, with antibody molecules establishing a more permanent bond with the egg surface than other proteins, as evidenced by centrifugation experiments (Tyler and Brookbank, 1956b). The inhibition by normal sera and BSA does mimic to some extent the inhibition of transport by p-chloromercuribenzoate (p-CMB) applied during the lag phase (Whiteley and Chambers, 1960). In concentration of 1.4×10^{-4} M, p-CMB virtually abolishes phosphate transport without inhibiting or delaying cleavage, a situation paralleled by normal sera and BSA. The effect of p-CMB is abolished by 10^{-3} M cysteine. The presence of at least some available cysteine residues in native proteins would seem to rule against anything similar in the mode of action of p-CMB and serum proteins. From the above information, it seems reasonable to conclude that: (1) phosphate uptake can be severely curtailed, without interfering with cleavage, by normal sera or BSA; (2) immune sera, diluted to the point where uptake approximates that of eggs in normal sera, continue to inhibit cleavage. Point (1) indicates a lack of dependence of early development on rapid uptake of phosphate. Point (2) would lead to the conclusion that cleavage inhibition by antisera against fertilizin does not result from decreased transport of phosphate.

Correlation of per cent inhibition of transport with nitrogen (protein) content of dialyzed sera or BSA is reasonably good only when BSA alone is considered, or when dilutions of a given serum are compared with one another (Table I, Experiment 11). In view of the complexity of mammalian sera and the lack of knowledge of the type of union between eggs and various serum proteins, it is not surprising that a more clear-cut relationship was not found. The possibility also exists of effects on P^{32} transport by non-dialyzable, non-nitrogenous serum compounds.

It is of interest that embryos in undiluted, sea-water-dialyzed, normal sera rarely, if ever, gastrulate. Lower concentrations of normal sera (0.5–1%) allow gastrulation but result in radially symmetrical larvae (Brookbank, unpublished data) with small skeletal spicules. In view of the lowered phosphate transport in the presence of serum proteins, one might postulate that lack of sufficient quantity of this ion ultimately may produce developmental abnormalities of primary mesenchyme metabolism and function during and after gastrulation.

SUMMARY

1. Normal rabbit sera and antisera prepared in rabbits against sea urchin fertilizin were shown to have an inhibitory effect on uptake of phosphate by

fertilized eggs. Immune sera caused more pronounced decreases in phosphate transport.

2. Bovine serum albumin solutions caused similar decreases in rate of phosphate transport.

3. Inhibitory effects of proteins were presumed to be due to a combination of protein with the egg surface, rather than a binding of phosphate by proteins in solution.

4. Antisera against fertilizin were without effect on phosphate uptake by unfertilized eggs.

5. The results are discussed in light of other works on egg activation and phosphate uptake.

LITERATURE CITED

Ballentine, R., and J. R. Gregg, 1947. The use of potassium biiodate in iodometric determination of ammonia. *Anal. Chem.*, **19**: 281–283.

Boell, E. J., 1945. Techniques for estimation of total nitrogen in tissues and in tissue extracts. *Trans. Conn. Acad. Arts Sci.*, **36**: 429–441.

Brookbank, J. W., 1959. The respiration of unfertilized sea urchin eggs in the presence of antisera against fertilizin. *Biol. Bull.*, **116**: 217–225.

Harvey, E. B., 1956. The American Arbacia and Other Sea Urchins. Princeton University Press, Princeton, New Jersey.

Krahl, M. E., 1950. Metabolic activities and cleavage of the egg of the sea urchin, *Arbacia punctulata*. A review, 1932–1949. *Biol. Bull.*, **98**: 175–217.

Litchfield, J. B., and A. H. Whiteley, 1959. Studies on the mechanism of phosphate accumulation by sea urchin embryos. *Biol. Bull.*, **117**: 133–149.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. Endall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

Palmer, L., 1937. The shedding reaction in *Arbacia punctulata*. *Physiol. Zoöl.*, **10**: 352–367.

Perlmann, P., 1957. Analysis of the surface structure of the sea urchin egg by means of antibodies. I. Comparative study of the effects of various antisera. *Exp. Cell Res.*, **13**: 365–390.

Perlmann, P., 1959. Immunochemical analysis of the surface of the sea urchin egg—An approach to the study of fertilization. *Experientia*, **15**: 41–52.

Tyler, A., 1949. A simple, non-injurious method for inducing spawning of sea urchins and sand dollars. *Coll. Net*, **19**: 19–20.

Tyler, A., 1959. Some immunobiological experiments on fertilization and early development. *Exp. Cell Res. (Suppl.)*, **7**: 183–199.

Tyler, A., 1963. The manipulations of macromolecular substances during fertilization and early development of animal eggs. *Amer. Zool.*, **3**: 109–126.

Tyler, A., and J. W. Brookbank, 1956a. Antisera that block cell division in developing sea urchin eggs. *Proc. Nat. Acad. Sci.*, **42**: 304–308.

Tyler, A., and J. W. Brookbank, 1956b. Inhibition of division and development of sea urchin eggs by antisera against fertilizin. *Proc. Nat. Acad. Sci.*, **42**: 308–313.

Tyler, A., A. Seaton and J. Signoret, 1961. Further analysis of antidevelopmental action of antisera against fertilizins and against other egg extracts of sea urchins. *Amer. Zool.*, **1**: 394.

Whiteley, A. H., and E. L. Chambers, 1960. The differentiation of a phosphate transport mechanism in the fertilized egg of the sea urchin. Symposium of germ cells and development. Institut Intern. d'Embryologie and Fondazione A. Baselli; pp. 387–401.

A MECHANISM FOR OBTAINING MATURE GAMETES FROM STARFISH[1]

ALFRED B. CHAET

Department of Biology, The American University, Washington 16, D. C., and Marine Biological Laboratory, Woods Hole, Mass.

The availability and use of marine gametes in biological studies has been an important part of scientific research for many years, and yet most investigators have utilized the gametes of relatively few species. For the most part, the animals (sea urchin, sand dollar, *Chaetopterus*, etc.) have been chosen because (1) they were readily obtained, (2) they were available in large numbers, and (3) their eggs were secured in large volumes and in a mature stage which exhibited synchronous division. Although starfish are available in large numbers, their gametes have not been used extensively (other than in studies of nucleoli) because mature gametes (exhibiting germinal vesicle breakdown) have been difficult or impossible to obtain precisely when needed. The standard method for securing "mature" gametes (Costello *et al.*, 1957) involves excising the ovaries from large, ripe animals. When these ovaries are placed in fingerbowls of sea water, eggs should exude through the cut end of the gonad. This technique leaves much to be desired, even if the experiment is performed on large, ripe starfish. In many instances the eggs obtained in this manner exhibit poor germinal vesicle breakdown, and one cannot always obtain successful fertilization.

The current report, which is a more detailed study than that reported in 1959 (Chaet and McConnaughy), describes a new method for obtaining starfish eggs (or sperm) and has the advantage of releasing only ripe, highly fertile eggs. In addition, if only a few cc. of eggs are required, small (2–3-inches) ripe starfish can be used and are available during the summer season at the Marine Biological Laboratory. This technique involves injecting reasonably ripe starfish with an extract containing "shedding substance" prepared from the radial nerves of donor starfish.

MATERIALS AND METHODS

In preparing extracts of shedding substance, large (8-inch) donor starfish, *Asterias forbesi*, were used. In most cases, these donor starfish (both male and female) had already spent their gametes prior to being brought into the laboratory.

In order to isolate the nerves, the arms from several starfish were severed just distal to the oral disc and placed, oral side down, in a dry pan until the arms flattened. The aboral surface of the arm was removed by making a lateral incision on each side of the arm. The oral half was quickly rinsed in sea water, and an incision, about 1 mm. in depth, was made along the entire length of the coelomic cavity side of the ambulacral groove. The two pieces, right and left, of the groove were carefully separated, and the radial nerve was seen attached to either one of the separated portions, or would frequently run from one to the other (Fig. 1). The nerve was then isolated with the aid of forceps and probe, and the entire nerve placed in a beaker of iced sea water. In this manner even an inexperienced worker can isolate all the radial nerves from 20 to 50 starfish in a few hours.

---

[1] This work was supported by grants from the National Institutes of Health and the National Science Foundation.

The isolated nerves were then washed three times in sea water (1 cc. per nerve), and once in ether. At this point, the nerves were then stored frozen until they could be dried by lyophilization. The dried nerves were then thoroughly pulverized into a fine powder, which will last indefinitely and from which homogeneous aliquots can be taken when needed.

Although lyophilization appeared to be the best way of releasing and storing the shedding substance, previous experiments (Chaet and McConnaughy, 1959; Chaet and Musick, 1960) have shown that the shedding substance could be released either by heating a sea water suspension of nerves (1½ minutes at 76° C.), or by lysing the nerves in distilled water. Once the nerves were lysed (2 cc. per nerve), salts were added before the extract was injected into starfish.

In vivo release of gametes from ripe starfish

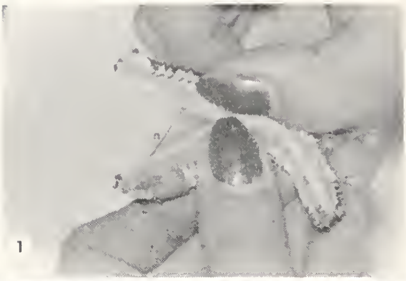
The choice as to the size of starfish from which to obtain gametes depended upon the time of year, the quantity of gametes required, and the amount of extract available for injection. Usually, small three-inch starfish (*Asterias forbesi*) were used. These small, ripe animals were made available by the Supply Department at the Marine Biological Laboratory during the entire summer. As has been noted, ripe starfish are virtually impossible to obtain in appreciable quantities during July and August. Once collected, the small starfish were iced and immediately brought into the laboratory where they were maintained in refrigerated (copper-free) running sea water (12° C.). The animals were fed live *Mytilus* to insure against gonad resorption. In this manner, 200 to 400 small starfish were maintained for weeks in their ripe condition, and additional supplies were obtained from the ocean when needed.

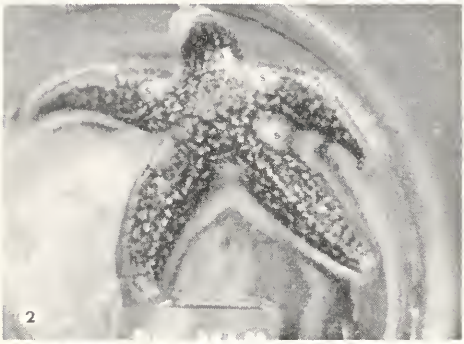
About one hour before a supply of gametes was required, a 1–5 mg.% sea water solution of the radial nerve powder was prepared. This sea water preparation could be stored indefinitely if frozen, but lost its activity if merely refrigerated. Ripe starfish were slowly injected, into the coelomic cavity, in a proximal direction from the distal end of an arm with 0.15 cc. of extract per gram of starfish (2–4 cc. per starfish). The injection required about 30 seconds for, if performed too rapidly, the dermal papillae would rupture. The animals were then kept in moist fingerbowls, or were inverted over beakers of sea water. Shedding usually began within 20 to 30 minutes after injection and would continue for 15 to 30 minutes thereafter, depending on the size and maturity of the gonads.

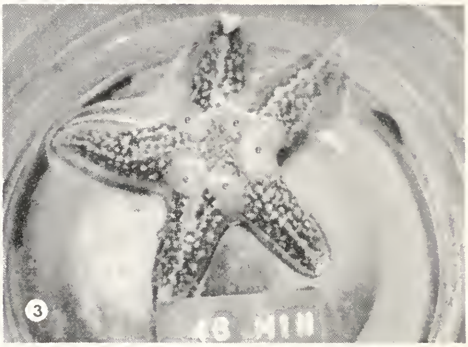
The eggs, if shed in a dry fingerbowl, were pipetted from the vicinity of the gonopores by flushing with a few cc. of sea water. They were then washed several times, as suggested by Costello *et al.* (1957). The sperm were kept dry and diluted prior to fertilization (Costello *et al.*, 1957).

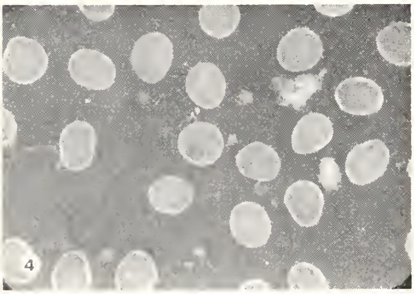
In vitro release of gametes from isolated ovaries

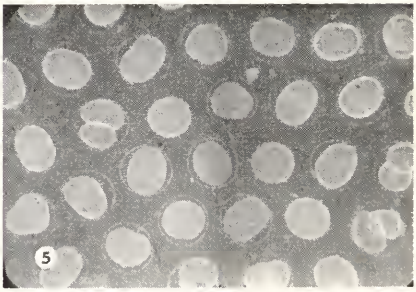
When individual batches of eggs were required from the same animal, all 10 ovaries were isolated and kept in dishes containing sea water, or placed in a 5 mg.%

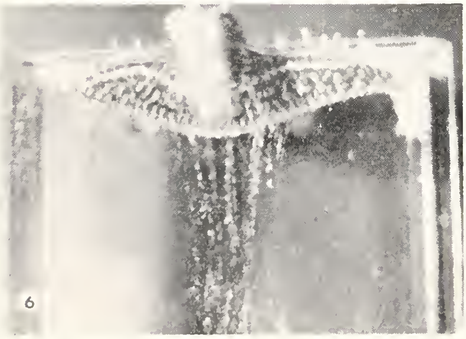












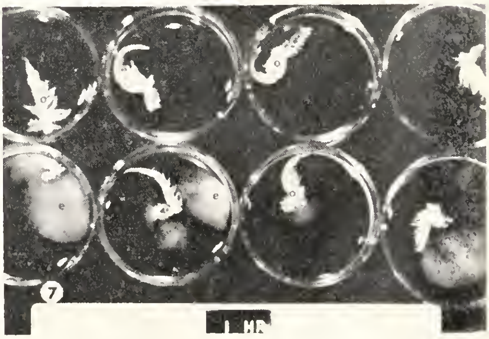


FIGURE 1. Photograph demonstrating isolation of radial nerve—the aboral surface of arm has been removed. Note the radial nerve complex (n) running from left to right.

FIGURE 2. Small (three-inch) recipient male starfish 30 minutes after injection of shedding substance (0.15 mm./cm.) (kept in a moist fingerbowl). Note accumulation of shed sperm in the vicinity of the gonopore (s).

solution of shedding substance. The ovary-gonoduct-gonopore preparation was easily obtained by removing the oral side of the arm after making a lateral incision along the entire length of both sides of the starfish arm. The aboral surface containing the two ovaries was then immersed in a bowl of sea water, and the arm (including the gonopore) was carefully pulled free from the oral disc. While the ovaries were suspended in sea water, a cut was made around each gonopore, thus isolating, intact, both ovary-duct-pore preparations from the arm. After washing these preparations in sea water, they were then immersed in either control or experimental solutions.

RESULTS

Table I illustrates the results of a typical experiment in which a group of experimental small, three-inch, ripe starfish were injected with 0.15 cc. of a 5 mg.% sea water solution of lyophilized radial nerves. Typically, 80–100% of the animals injected with nerve extract began shedding their gametes in about 30 minutes, whereas none of the ripe starfish injected with sea water (controls) shed their gametes. The volume of gametes shed (quantity shedding) has been noted, and each rating (0 to +5) is related to the overall size of the animal injected (Table I). The size and condition of the gonad observed upon autopsy were also noted (Table I, gonad value).

The release of gametes in experimental animals (30 and 46 minutes after they have been injected with a solution of shedding substance) is illustrated in Figures 2 (male) and 3 (female). Once injected, the animals were kept in moist fingerbowls. Although starfish began to shed at about 30 minutes, appreciable quantities of gametes were not expelled for some minutes later. The animal in Figure 2 was considered to be in a #2 stage of shedding (#5 would denote maximum shedding), whereas Figure 3 was considered a #5 stage. The eggs, once collected from the aboral surface of the moist starfish, exhibited a high degree of germinal vesicle breakdown (98–100%) and were highly fertile (95–97% exhibited elevated fertilization membranes). Photomicrographs of unfertilized and fertilized eggs obtained by this technique are shown in Figures 4 and 5. Similarly, highly fertile eggs were obtained when heated or lysed nerve extracts were used, although it should be pointed out that substantially more shedding substance was found in extracts when they were prepared from lyophilized nerves.

Figure 6 illustrates an animal that was inverted over a container of sea water

FIGURE 3. Small (three-inch) recipient female starfish, 46 minutes after injection of shedding substance. Note accumulation of eggs (e) at all 10 gonopores.

FIGURE 4. Photomicrograph of a representative field of washed, diluted, but unfertilized starfish eggs obtained after injection of shedding substance. Note high percentage of germinal vesicle breakdown.

FIGURE 5. Photomicrograph of a representative field of fertilized starfish eggs obtained after injection of shedding substance. Note the high percentage of fertilization membranes and cells beginning to undergo cleavage.

FIGURE 6. Small (three-inch) recipient starfish, inverted over a container of sea water, 30 minutes after injection of shedding substance. Note the streams of eggs dropping from the 10 gonopores.

FIGURE 7. Dishes containing 8 ovaries from the same starfish—the ovaries (o) in upper row immersed in sea water for one hour—lower row, in 5 mg.% shedding substance for one hour. Note release of eggs (e) in the form of a cloudy mass in only those ovaries in shedding substance.

TABLE I

Typical experiment showing the results of an injection of shedding substance into the coelomic cavity

Three-inch ripe *Asterias forbesi* injected (0.15 cc. gm) with:

Control (Sea water)				Experimental (Nerve shedding substance)			
Shed	Gametes	*Quantity shedding	**Gonad value	Shed	Gametes	*Quantity shedding	**Gonad value
no	—	0	+3	yes	female	+4	+2
no	—	0	+1	yes	male	+1	+1
no	—	0	+3	no	–	0	+1
no	—	0	+1	yes	female	+1	+2
no	—	0	+3	yes	female	+3	+1
no	—	0	+3	yes	male	+3	+2
no	—	0	+1	no	–	0	+1
no	—	0	+2	yes	male	+2	+3
no	—	0	+3	yes	male	+4	+4
0% shed				80% shed			

* #1 given to an animal which exhibited minimum shedding of gametes, whereas #5 indicates maximum shedding.

** Refers to the size and condition of the gonad seen upon autopsy; #0 given to a barely visible gonad, whereas #5 would indicate a large gonad filling most of the coelomic cavity.

and was in the process of releasing several streams of eggs, which can be seen falling to the bottom of the container. Note that all gonopores released eggs, suggesting that all 10 ovaries have been stimulated to shed by the injection of shedding substance. This method of releasing eggs directly into sea water is advantageous to those who require that the eggs be placed in a particular solution immediately upon discharge. An *in vitro* experiment in which 8 ovaries from one starfish were isolated and placed in sea water is illustrated in Figure 7. At 0 time, four ovaries were immersed in a solution of shedding substance (5 mg.%) (bottom row), and the other four ovaries (top row) in fresh sea water. Note that only those ovaries in shedding substance released any eggs (cloudy mass). If the ovaries were not excessively handled during isolation procedures, they would usually shed simultaneously. The precise onset of shedding (26 vs. 35 minutes) appeared to be a function of the particular animal, and within a given starfish, all 10 ovaries began to shed at approximately the same time.

DISCUSSION

As can be noted from Table I, a starfish injected with shedding substance will release mature gametes in about 30 minutes. In those instances when the animal does not respond to an injection of shedding substance, subsequent autopsy has invariably shown that the gonads were extremely small and immature. Although, in the experiments described in this paper, animals were carefully weighed and a precise dosage of shedding substance injected, this step is not necessary for those investigators merely wishing to obtain an adequate supply of eggs. The exact quantity of extract injected need not be precisely measured, since experience has shown that there is ample leeway in this regard, and that one can approximate the amount required for injection rather than weigh each animal.

The successful use of this technique in the hands of other investigators since the original publication of this method (Chaet and McConnaughy, 1959; Chaet and Musick, 1960; Chaet and Rose, 1961; Chaet and Smith, 1962; Hartman and Chaet, 1962) has been recently reported not only for *Asterias forbesi* (A. Monroy, personal communication), but for species from the Japanese coast as well (Noumura and Kanatani, 1962).

When the *in vitro* experiments involving isolated ovaries were performed, care was taken not to obstruct, pinch, or damage the gonoduct or gonopore area. If this exit was pinched, shedding did not begin at the anticipated time (30 minutes) but was delayed for an additional 10–15 minutes. Under these conditions, the eggs were still extruded and were mature, but were released only through a break in the gonoduct rather than being shed through the gonopore itself.

It is interesting to point out for those wishing to use this echinoderm material throughout the summer months that the length of the breeding season of *Asterias forbesi*, and thus the time that eggs can be available for experimental studies, has been prolonged by collecting a supply of small, ripe starfish and storing them in the laboratory in refrigerated running sea water. If the animals are fed and held at lower temperatures, they will continue to contain mature gonads, even though those taken from the ocean have shed in their natural environment.

Since this paper deals with the method of obtaining gametes from starfish, no attempt will be made here to include the physiological or chemical nature of the shedding substance, which will be reported elsewhere.

SUMMARY

A physiological method of releasing gametes from ripe starfish, *Asterias forbesi*, has been described. The recipient starfish responds within 30 minutes to an injection of 5 mg.% of dried, lyophilized radial nerve extract by releasing eggs exhibiting a high degree of germinal vesicle breakdown, which are highly fertile. The shedding substance acts directly on isolated ovaries, thus enabling simultaneous experimentation on 10 gonads from the same female.

LITERATURE CITED

CHAET, A. B., AND R. A. MCCONNAUGHY, 1959. Physiologic activity of nerve extracts. *Biol. Bull.*, **117**: 407–408.

CHAET, A. B., AND R. S. MUSICK, 1960. A method for obtaining gametes from *Asterias forbesi*. *Biol. Bull.*, **119**: 292.

CHAET, A. B., AND R. A. ROSE, 1961. Further studies on the gamete "shedding substance" from radial nerves. *Biol. Bull.*, **121**: 385–386.

CHAET, A. B., AND R. SMITH, 1962. Role of gamete-shedding substance from starfish nerves. *Amer. Zool.*, **2**: 213.

COSTELLO, D. P., M. E. DAVIDSON, A. EGGERS, M. H. FOX AND C. HENLEY, 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Marine Biological Laboratory, Woods Hole, Mass.

HARTMAN, H. B., AND A. B. CHAET, 1962. Gamete shedding with radial nerve extracts. *Fed. Proc.*, **21**: 363.

NOUMURA, T., AND H. KANATANI, 1962. Induction of spawning by radial nerve extracts in some starfishes. *J. Fac. Sci. Univ. Tokyo, Ser. IV*, **9**: 397–402.

ADAPTIVE ASPECTS OF ACTIVITY RHYTHMS IN BATS[1]

GEORGE DeCOURSEY AND PATRICIA J. DeCOURSEY

Zoophysiological Institute, University of Tübingen, Germany, and the Zoology Department, University of Wisconsin, Madison 6, Wisconsin

A daily cycle of activity in animals was first noted centuries ago, but only in the past decade has substantial progress been made in analyzing the complex interaction of physiological and environmental regulatory factors. Research in circadian physiology indicates that an endogenous timing system, coupled with some daily recurring agent of the environment, provides the basis for activity rhythm regulation of many species (Aschoff, 1958, 1960; DeCoursey, 1961; Pittendrigh, 1960). For some of the remaining problems, bats afforded several unique and promising approaches. Since bats are strongly nocturnal, but generally depend upon auditory rather than visual stimuli (Griffin, 1958), they were of particular interest for determining the effectiveness of the daily light cycle as an activity synchronizer. Furthermore, their preference for roosting in caves, and dimly lit buildings, provided opportunities for studying environment-testing behavior, and for demonstrating the ecological value of circadian rhythms. Results are presented here for experiments in the laboratory and in natural habitat.

The research was carried out under the auspices of the Zoophysiological Institute of the University of Tübingen, Germany; it is a pleasure to acknowledge the generous assistance of the Director, Professor F. P. Moehres. We extend our thanks also to the Institute's staff for help with many phases of the work; to Dr. Rolf Gögel, Pastor in Wendelsheim, Germany, for his hospitality and cooperation which made possible the studies of the *Myotis myotis* colony; to Professor Jürgen Aschoff of the Max-Planck Institute, Erling, Germany, for making available recording apparatus; to Professor Leo Pardi of the University of Turin, Italy, for help in collecting the *Rhinolophus;* and to the Zoology Department of the University of Wisconsin, especially to Professor John Emlen, for furnishing working accommodations during the preparation of the manuscript. Dr. James King and Dr. Horst Schwassmann gave helpful suggestions for the manuscript. Financial support was granted to the senior author through Public Health Fellowship No. BF-11,999 from the National Institute of Neurological Diseases and Blindness, of the United States National Institutes of Health.

Part I: Experimental laboratory studies

Material and methods

The greater European horseshoe bat, *Rhinolophus ferrum-equinum*, can be maintained in small rooms over long periods of time in good health, and its daily activity accurately recorded without restriction of the animals to cages. Experiments were based upon three individuals collected from a cave in hibernating condition. In the laboratory the bats were first trained to fly to a screen-wire platform to obtain mealworms and water from small dishes.

---

[1] Present address: Zoology Department, Washington State University, Pullman, Washington.

Two smooth-walled, light-proof rooms were used in measuring activity rhythms of individual bats. Temperatures were held as uniform as possible (Figs. 1 and 2). Light schedules were provided by a 40-watt incandescent bulb and an electric timer. The rooms were entered at irregular intervals of 1–10 days for changing food and water (indicated on Figures 1 and 2).

The recording method in these two rooms took advantage of the free-hanging roosting habit of *Rhinolophus*. A string perch, used consistently by a bat, was

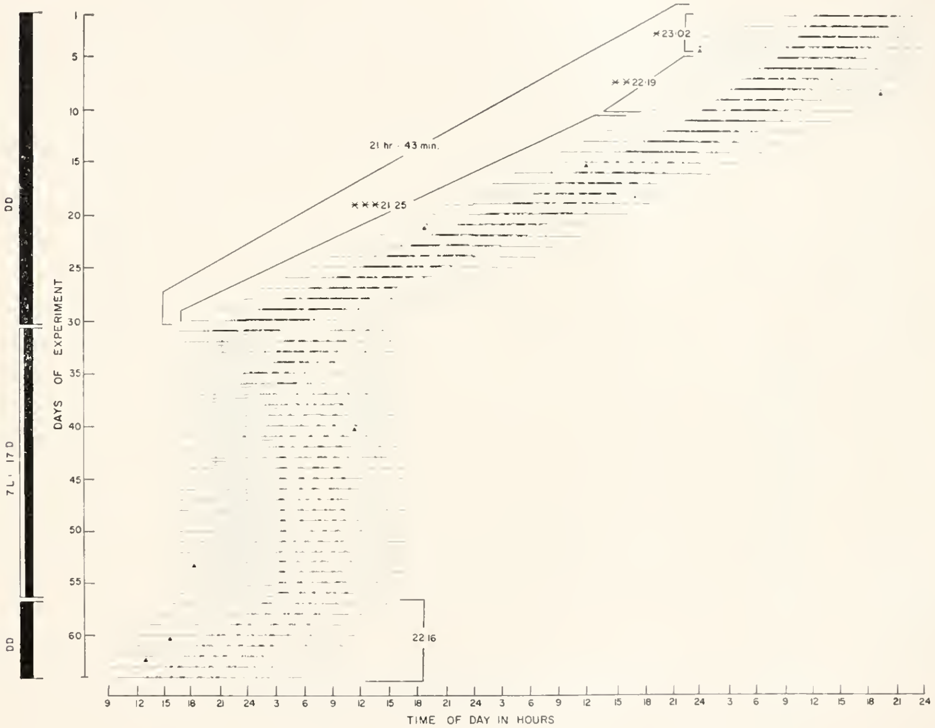


FIGURE 1. Light synchronization experiment with *Rhinolophus* #2: DD on days 1–30 → 7L:17D on days 31–34 → DD on day 35 → 7L:17D on days 36–56 → DD on days 57–64. Note day 35 in DD to distinguish masking effect of the light schedule. Nomenclature in the figures and text follows Pittendrigh (1960): DD, continuous darkness; *x*L:*y*D, a light schedule with *x* hours of light and *y* hours of darkness. Each single mark of the recorder pen represents a flight from the roosting string and return; blocks indicate frequent, short flights. Light during the artificial day schedule is shown by underlining, feeding in the light by △, and feeding in the dark by ▲. Temperature range: 22°–27° C., usually less than 2° C. fluctuation per day. Cycle length of the rhythm in DD is indicated by bracketed numbers, with the changing cycle length on days 1–30 emphasized by the three subdivisions *, **, ***. Extended time scale and splicing of the record are used for convenience in studying the frequency. For further explanation see text.

attached to a microswitch for registering flight and rest activity on an Esterline-Angus Operations Recorder. Daily segments were subsequently mounted in chronological order for graphic portrayal (Figs. 1 and 2).

A third light-proof room housed a large, darkened, artificial bat cave. The cave was a U-shaped plywood tunnel with arms 3.7, 2.1, and 1.3 meters in length, respectively, and 0.6 by 0.6 meters in cross-section. At the closed end a partial partition created a small chamber where the bats could hang from a screen-wire grid. All other footholds in the tunnel were excluded and the entire interior was light-proofed except for the open end, then painted flat black for maximum darkening. Temperature varied between 21° and 26° C., usually less than 2° C. per day. A 40-watt overhead incandescent light and control clock supplied the room light schedule. Food and water were available *ad libitum* outside the tunnel,

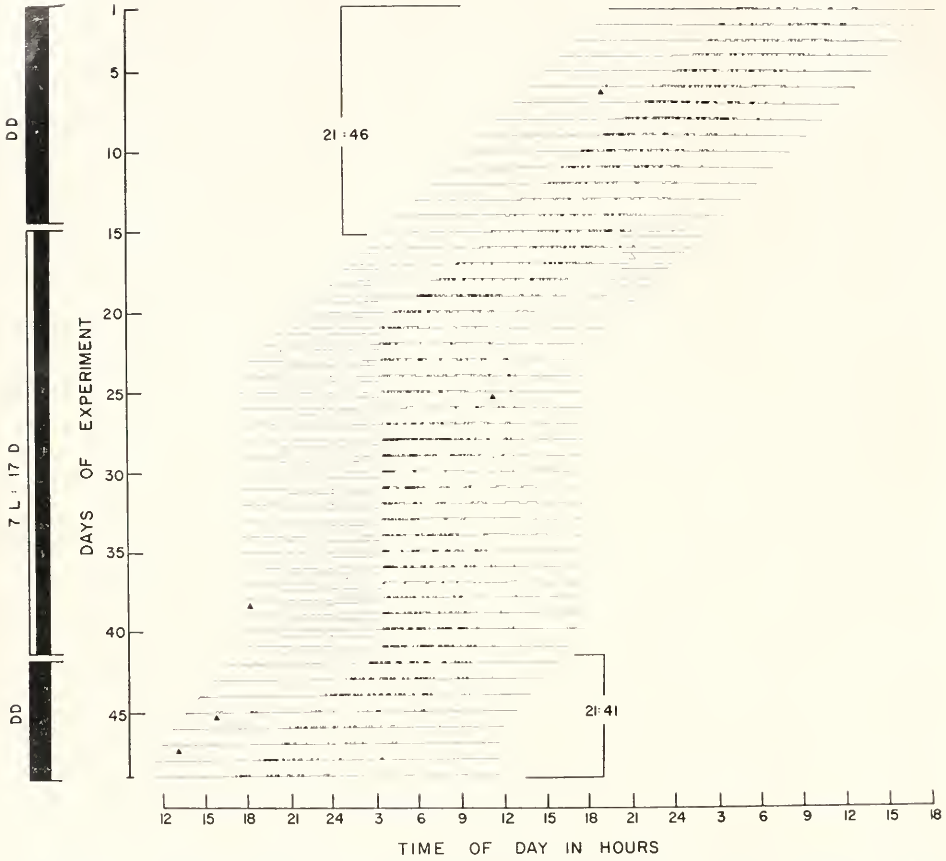


FIGURE 2. Light synchronization experiment with *Rhinolophus* #1: DD on days 1–14 ⟶ 7L:17D on days 15–41 ⟶ DD on days 42–48. Temperature range *normally* 21°–27° C. with about 3° C. variation per day; elevated baseline reflects failure of room temperature regulation, during which time the bat hung for short periods of time from a cool air vent. Other symbols as in Figure 1.

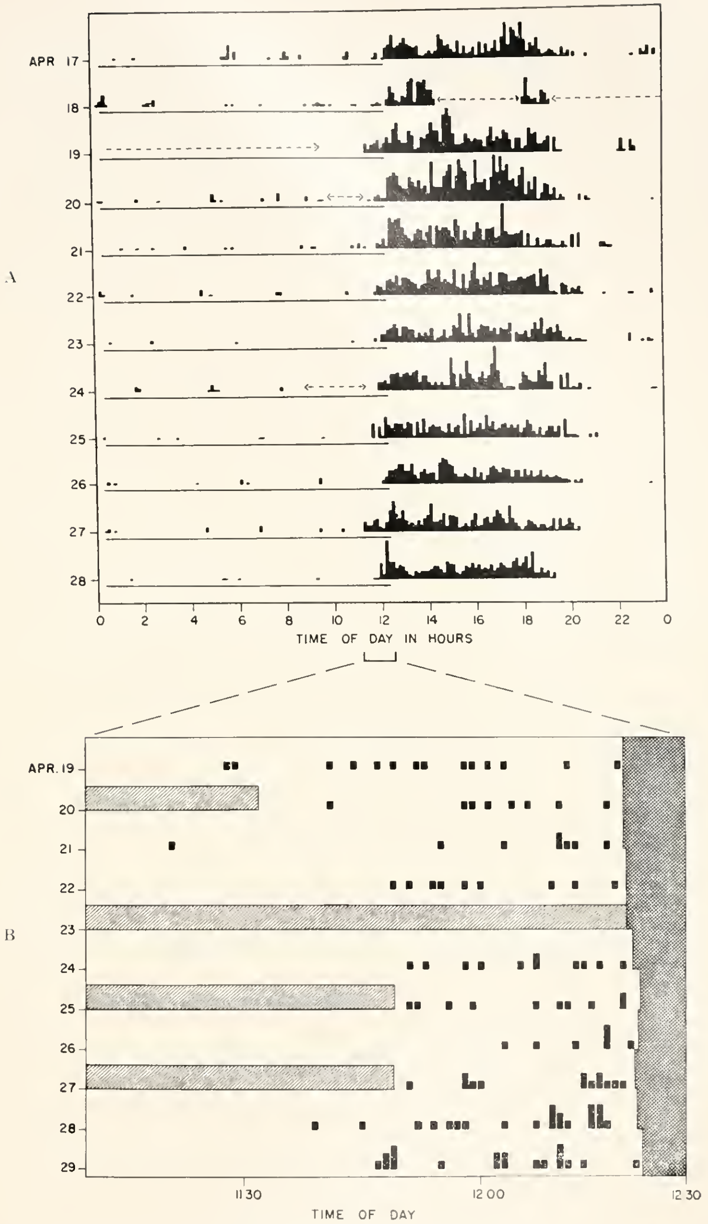


FIGURE 3. Artificial bat cave experiment, showing flight activity of *Rhinolophus* #1, 2, and 3 with constant temperature and lighting in cave, and 12L:12D in room. A. Activity from April 17–28, 1961, plotted as photocell counts/8 minutes; underlining for light hours in the room, and broken line for equipment failure. B. Activity during the time of day bracketed in A, from April 19–29, 1961, graphed as number of flights per minute up to or beyond the end of the tunnel; lack of observation shown by single hatching (no observers), or double hatching (darkness). For further explanation see text.

and were renewed by entering the room during the light period about every 5 days.

Under these conditions, the flights of the bats along the tunnel could be recorded by a dim white light beam and photocell unit near the roosting chamber. The beam was visible to a bat, while roosting, as a continuous glow, but gave no information about the day-night schedule outside the tunnel. Number of flights was tabulated on an electric counter outside the room, then photographed with an automatic camera device. An infra-red photocell unit recorded the number of flights past the open end of the tunnel; the counter was read directly for certain experiments (Fig. 3B).

Observations

A strong tool for defining which cyclic, exogenous factors are able to control the phase of an endogenous rhythm has been to change an animal from a constant environment to one in which a single factor fluctuates with 24-hour periodicity. In the first experimental series of this study, a single horseshoe bat was confined to each of the two small recording rooms, and activity first measured in DD, then in L:D, and finally again in DD (for terminology see Figure 1). The responses of the two bats (Figs. 1 and 2) were similar to those reported for a number of other nocturnal species (Aschoff, 1960; Bruce, 1960; DeCoursey, 1961, 1963; Justice, 1960; Pittendrigh, 1960; Rawson, 1959; Roberts, 1962; Stewart, 1962). The results are characterized by (1) a persistent, non-24-hour rhythm of activity in the absence of a light cycle, (2) the gradual adjustment of the time of activity during the light schedule, and (3) the ultimate establishment of a nocturnal activity pattern.

Several points merit special emphasis. The envelope of the active period, as well as the clear starting point, demonstrates the circadian nature of the rhythms in darkness. In one case the active period scanned the entire solar day in the course of several weeks (Fig. 1, top). Both inter- and intra-individual variation occurred (Figs. 1 and 2). The spectrum for these few measurements of mean cycle length (plus two additional values not shown) encompasses values from 21 hours:49 minutes to 23 hours:27 minutes. These fall in the range of values shown by comparable measurements for several species of bats (Griffin and Welsh, 1937; Menaker, 1961; Pohl, 1961; Rawson, 1960), but are much shorter than for most other nocturnal species (Aschoff, 1958; DeCoursey, 1961, 1963; Johnson, 1939; Justice, 1960; Pittendrigh, 1960; Rawson, 1959; Roberts, 1960; Stewart, 1962). Furthermore, the cycle length in any one test period may gradually change. The lability of the rhythm is particularly marked for *Rhinolophus* #2 (Fig. 1, top); also see Rawson (1960). Such a phenomenon has been observed by Menaker (1961) for bats, as part of the brief transition from the winter to the summer condition, but in this study the bats remained under the experimental conditions for many months. A variable frequency for free-running rhythms probably represents one end of a scale, ranging from the relatively stable rhythms of *Glaucomys* (DeCoursey, 1961), through the history-dependent rhythms of hamsters, finches, and cockroaches (Pittendrigh, 1960), to the extremely variable rhythms of *Muscardinus*, which may alter the period from about 25 to 20 hours (DeCoursey, unpublished experiments). The values for free-running rhythms of bats give an estimate of the magnitude of correction needed to synchronize the activity to the appropriate part of the day. The cycles of light and dark (Figs. 1 and 2, center) sufficed to bring about the necessary correction. In both instances nocturnality was established, but, as in other species, the pattern of adjustment depended upon the relationship of light to the activity of the animal (see DeCoursey, 1961, 1963 for a discussion of this point). Nocturnal activity has also been noted for two other species of bats under laboratory conditions (Griffin and Welsh, 1937; Kowalski, 1955).

The demonstration of rhythmic activity deviating slightly from 24 hours in cycle length, for bats in a constant environment, and the subsequent locking of the phase in an L:D regime was considered conclusive evidence for regulation of activity by both endogenous and exogenous factors. Whether cyclic factors other than light are significant has not yet been extensively investigated for bats. Griffin and Welsh (1937) suggest that feeding clues may be important for some individuals, in the absence of light clues, but Kowalski (1955) found no evidence for such a conclusion.

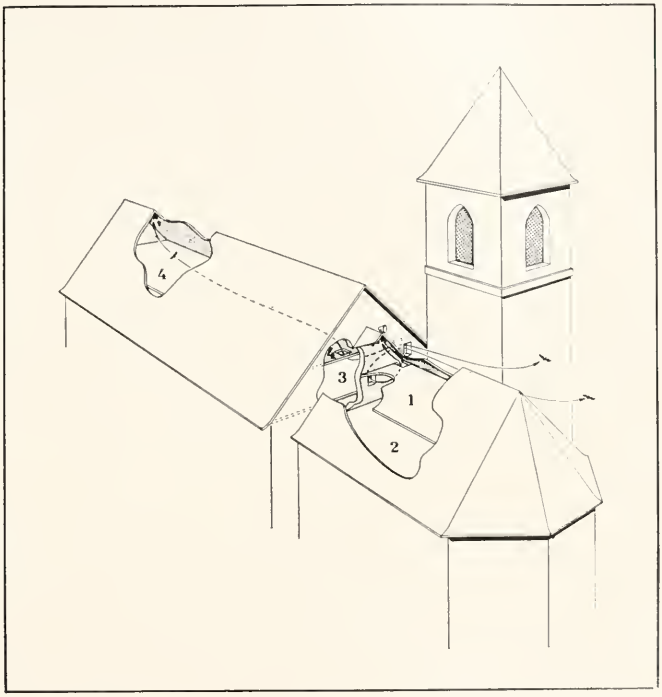


FIGURE 4. Cutaway diagram of the Wendelsheim loft. Single lines mark flight routes from roosting areas in Rooms 1 or 4 to outside of church, with broken line for that part of route not visible in diagram. See text.

As a consequence of the above experiments, the question arose as to the manner in which the bats detected the environmental changes to which they would eventually synchronize their activity. In the second series of experiments, the three horseshoe bats were placed in the artificial cave room for several months with room light on at midnight and off at noon. Preliminary observations revealed that the bats hung during the daylight hours almost exclusively in the darkened roosting chamber, then during the hours of darkness flew frequently back and forth through the tunnel. Since it was necessary for a roosting bat to fly the length of the tunnel to see the room light and gain access to the room, it was possible to determine the time of day at which such light-sampling took place.

The rate of activity, measured by the total number of flights past the counting cell per 8-minute interval, was a convenient indicator of the activity pattern. As in the first experiments, the bats remained inactive for most of the day. A few flights were made from the cave into the lighted room. All available evidence suggests that a bat, in at least the majority of cases, returned almost immediately to the roosting chamber, and therefore was exposed only momentarily to the light. The number of flights increased during the last hour of daylight, then rose sharply soon after lights out. Flight activity continued almost unabated for approximately 8 hours, tapering off well in advance of daylight (Fig. 3A).

The crucial point to note is the anticipation of the light change by these bats after the long daylight inactivity. In order to determine more exactly the number and duration of flights within the tunnel and in the room at this time, the bats and the electric counters were watched simultaneously for ½–3 hours preceding lights out, for several weeks (Fig. 3B). Usually the bats flew out into the room and circled once or twice before returning to the cave. In some cases the bats did not leave the tunnel, but turned before reaching the open end and flew back into the roosting chamber. In sharp contrast was the abrupt onset of activity for the bats in the first experimental series just after the lights out transition (compare Figures 1 and 2 with Figure 3B). The significance of these data is considered in the Discussion.

Part II: Field studies of activity in summer bat colonies

Material and methods

A summer colony of about 500 adult female *Myotis myotis* and their young was observed from June 24 to July 30, 1961, in the loft of the Wendelsheim church, Germany. Additional data were gathered from seven breeding colonies of *Myotis lucifugus* during the summer of 1962 in Madison, Wisconsin.

At the Wendelsheim loft, large sheets of transparent plastic were gradually lowered into place in order to channel the majority of the bats, in their departure and return, to a series of rooms having a pronounced light gradient (from roosting place in Room 1 to Room 2 to Room 3: see Figure 4). A secondary flight route between Rooms 4 and 3, used occasionally by a few bats, remained unchanged and was not usually observed. From the large central loft (Room 3), all bats exited through one small window, then flew down a narrow V-shaped trough formed by the tower wall and the nave roof before gaining a free flyway (Fig. 4).

From Room 3 it was possible to count departures and returns by watching the bats silhouetted through the open window against the sky. The beginning of activity in the dimmer roosting rooms was judged by direct observation, or by counting the rate of high-frequency calls of the bats at the small aperture between Rooms 2 and 3, using a microphone and oscilloscope detector (Fig. 5). At regular intervals during the evening flights, the light intensity outside the church was

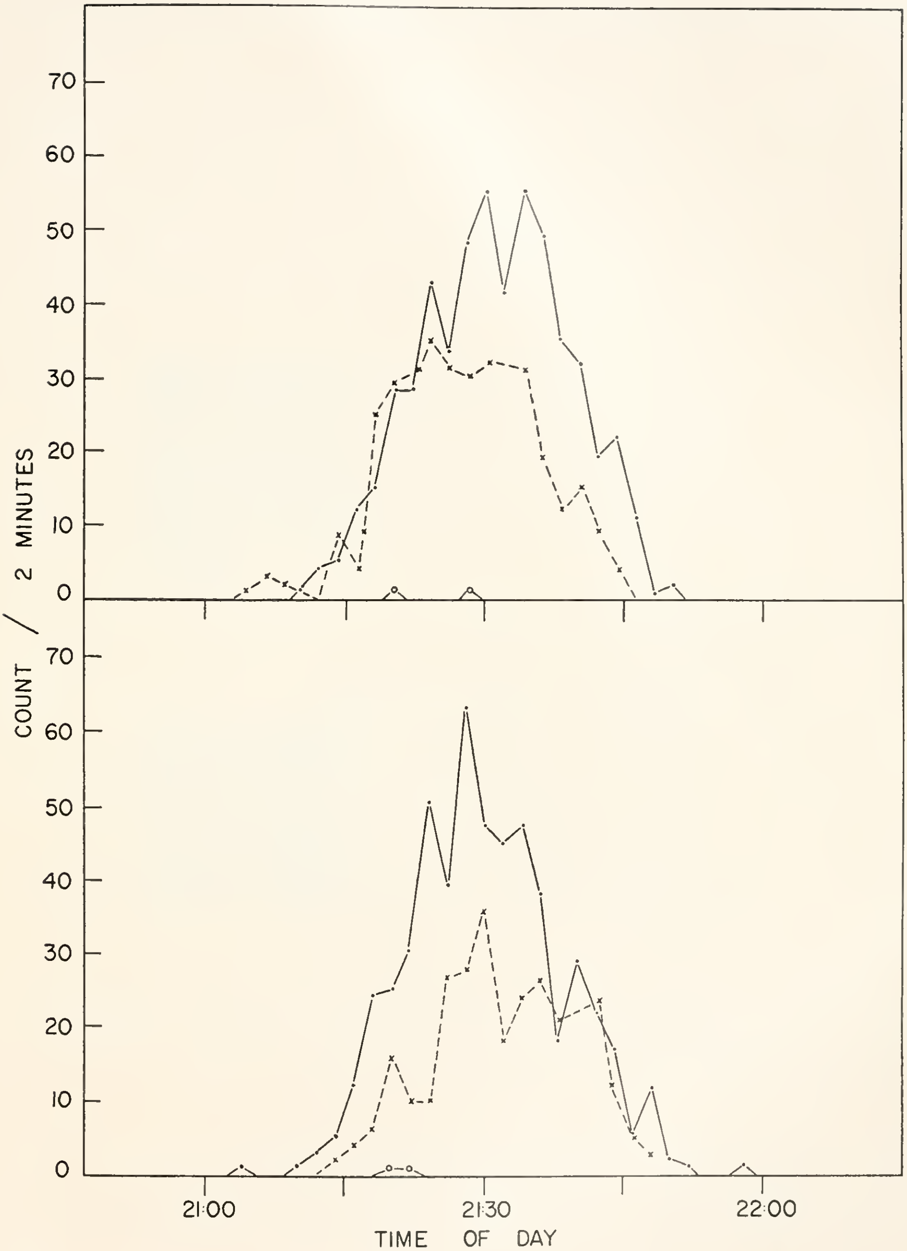


FIGURE 5. Evening activity of bats on two consecutive clear nights at Wendelsheim, July, 1961: • — • exit flights as bats/2 minutes, o — o returns to loft, x---x pre-exit flight activity estimated as the number of high-frequency calls/2 minutes at passageway from Room 2 to 3.

measured with a Lange Luxmeter. Simple recording methods were devised for counting and tabulating data *without light or noise disturbance to the bats.*

Observations

In spite of severe limitations, field studies offer possibilities for further insight into the problem of environment-sampling by the bats. Due to conspicuous colonial habits, bats are more suitable for surveillance than most nocturnal species, and have been the object of many observations. Emergence of bats from roosting places usually takes place at dusk (Allison, 1937; Church, 1957; Eisentraut, 1952; Moebus, unpublished data; Schwassmann, personal communication; Twente, 1955; Venables, 1953), often correlated with a small range of light intensities (Twente, 1955; Venables, 1953), but the darkness of a total eclipse during daytime did not evoke activity (Krzanowski, 1959). Exit flight times roughly parallel the time of sunset throughout the summer (Church, 1957; Moebus, unpublished data), thus implicating light as the chief synchronizer, in spite of the poorly developed vision of bats.

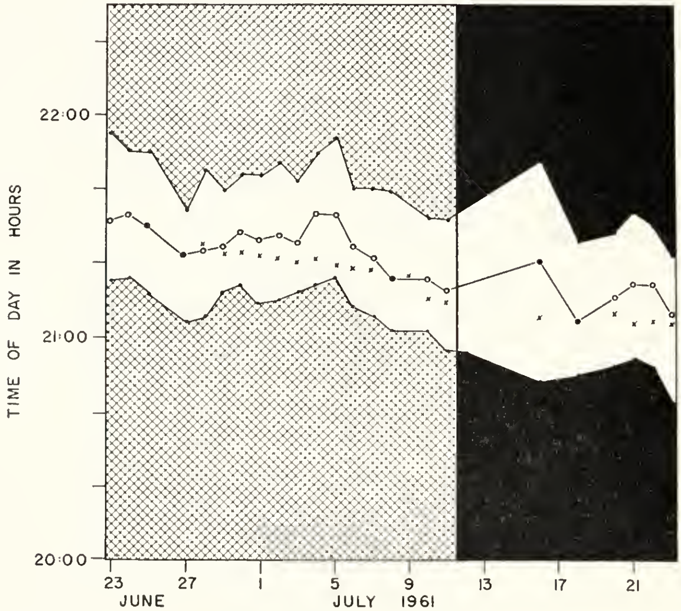


FIGURE 6. Summary for the Wendelsheim colony with white central zone indicating the flight time (exit count rising above four bats/2 minutes to count dropping below four bats/2 minutes) for the normally lighted loft at left, crosshatched, and for the darkened church at right, black. Symbols: o average exit time on clear days, and • on cloudy days, x for outdoor light intensity of 0.08 lux.

The Wendelsheim colony was also strictly nocturnal. After remaining relatively quietly in Room 1 or 2 during the day, the bats departed rapidly from the church about one-half hour after sunset. The flight rate rose in a few minutes to a peak, then declined until only the young and a few adults were left in the loft (Figs. 5 and 7). Little more than an hour elapsed between the first flight activity in the roosting loft and the end of the exit flight, and the actual departure of the 500 bats usually required less than 45 minutes (Figs. 5–7). Only occasional adults were seen until shortly before dawn; then the return flight into the loft lasted

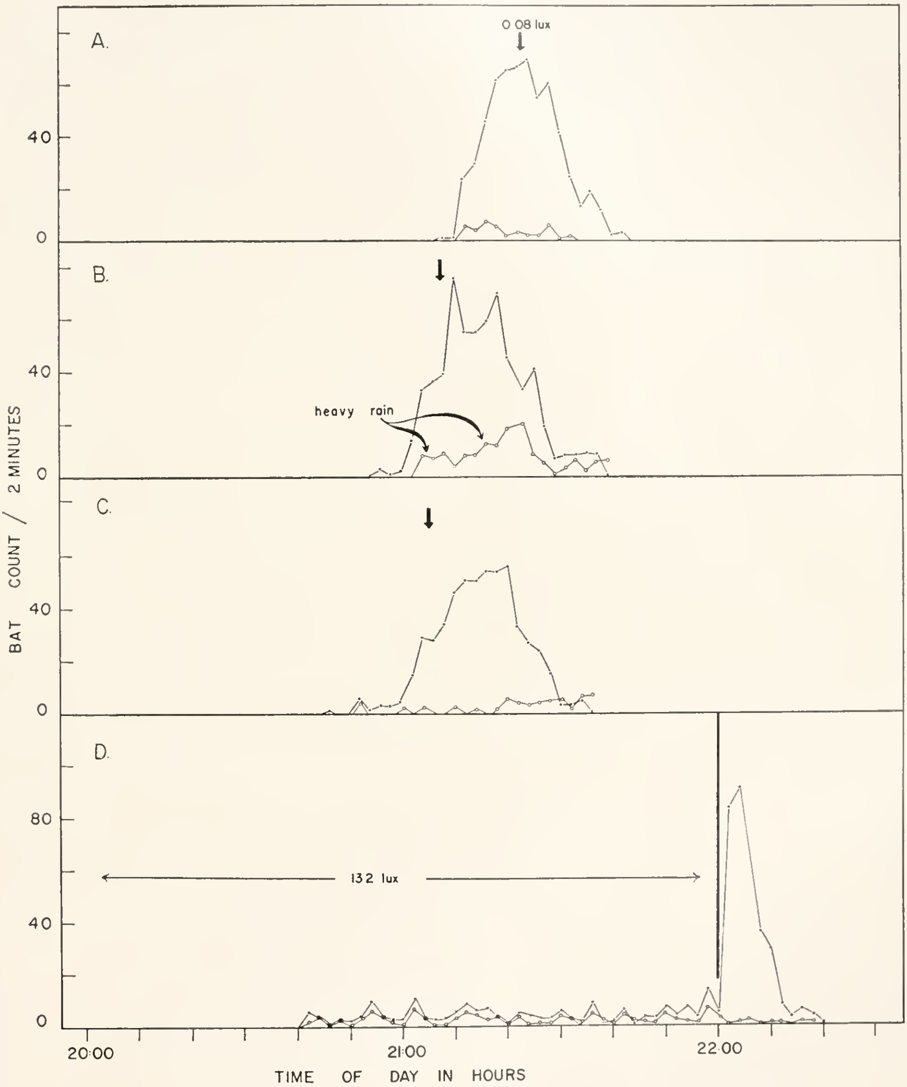


FIGURE 7. Environmental influences on activity at the Wendelsheim colony: A. Clear night in July, normal lighting. B. Rainy night in July, normal lighting. C. Clear night in August, darkened church. D. Illuminated flyway in August. Symbols: •—• exits, o—o returns, for outdoor light intensity of 0.08 lux.

slightly longer than the evening flight. Similarly, in Madison, great numbers of bats left attic roosting retreats in many old houses, and flew towards the lake in search of food. Due to the synchrony of the exiting bats, it was possible to locate several new colonies in a single evening merely by following the stream of bats at dusk back to the source. The counts at several colonies showed a pattern almost identical to the Wendelsheim flights, illustrated in Figure 5.

These observations also illustrated the close link between activity time and specific light intensities. In the normally lighted loft at Wendelsheim *average exit time* took place between 0.02 and 0.10 lux, with a mean value of 0.05 lux. No light meter was available in Madison, but on heavily overcast days the flight started five or ten minutes earlier. With the shortening days in July and August, the flights also began earlier, both at Wendelsheim (Fig. 6) and in Madison.

Other exogenous factors had only a minor influence. Daytime restlessness inside the Wendelsheim loft increased on hot, sunny days. Heavy rain often thoroughly disrupted the evening flight (Fig. 7B). After July 11, the young bats, recognizable by their uncertain flying, left with the adults but soon landed on the exterior walls; many crawled back into the church through the window. The increasing number of returns after mid-July, during or shortly after the exit flight, was attributable to the young (Figs. 7A and 7C). Thus, several meteorological and biological factors influenced the amount of activity to a small extent, but light seemed the chief synchronizer of activity throughout the season.

The sampling behavior (Twente, 1955) of the bats prior to the exit flight was apparently the means of testing the light intensity. Soon after leaving the roosting room at the Wendelsheim colony, the bats flew towards the exit window, often hovering there momentarily. The circling continued until one bat darted out the window, followed in rapid succession by many others (Fig. 5).

In order to further evaluate the importance of light sampling in a colony under natural conditions, an experiment was conducted at Wendelsheim. In the loft rooms, as many light leaks as possible were covered. This darkening and the initial structural changes (see Material and Methods) essentially duplicated the laboratory cave experiments, by requiring a bat, after arousal, to fly through the series of rooms to the exit window, to see if conditions were propitious for flight. The light intensity in the loft was reduced many-fold, but it unfortunately proved unfeasible to eliminate all the minute light leaks between the roof tiles. Neither earlier activity in the roosting room, nor earlier sampling resulted, nor was the timing of the exit flight affected (Figs. 6 and 7C). A second experiment was conducted on one evening, several weeks later. The V-shaped trough just outside the exit window was illuminated with a powerful floodlight (Fig. 4), from one-half hour before the usual start of the exit flight to one hour after expected peak exit time. As the normal departure time arrived the sampling became very intense and continuous, with large numbers of bats hovering near the window. A few flew out into the bright area but 67% re-entered the loft, most of them immediately. More adequate lighting of the outside of the church would probably have totally prevented exits. After lights out, the bats in the loft whirled and circled for a few seconds and then poured out through the window (Fig. 7D). In one of the few parallel field experiments, Hodgson (1955) was able to delay the departure of ants from an underground nest by covering the approaches at dawn with dark awnings, or conversely, by hanging a lantern above the nest before dawn, caused an early departure.

Discussion

The hypothesis that the daily activity of an animal depends upon an endogenous timer and clues of the environment is not peculiar to bats. In one sense, this example merely broadens the comparative base of a well grounded theory (significant examples or reviews in Aschoff, 1963; Bruce, 1960; Bünning, 1963; DeCoursey, 1960; Pittendrigh, 1960; Rawson, 1959). Bats were useful, however, for considering how an animal compensates for an endogenous timer which chronically runs too fast or too slow, and what useful purpose such built-in variability serves.

The exogenous clues are the sensory stimuli which set in action processes for holding the activity rhythm in appropriate phase with the environment. A synchronizer in its simplest form consists of two intensity extremes with transition zones between the two: for most animals a daytime condition of high light intensity followed by an abrupt twilight change to a dim night state, and a return to the daytime state. Sensory input may be dependent upon one or both transitions or upon the steady-state light intensity. These alternatives are over-simplifications for the sake of clarity, since effective synchronizers are known, ranging from those with instantaneous changes between the two steady-states, to near sinusoidal changes from maximum to minimum intensity (Swade, unpublished data). Furthermore, both continuous action and transitional type signals may contribute.

The present work gives little information on the time of actual correction of an endogenous element (see Aschoff, 1963 for further discussion of this problem). The laboratory and field experiments imply, however, that bats receive their sensory input primarily from the dusk transition of the environment. The animals living in the cave anticipated the light change and flew out briefly to sample. In contrast, the same individuals, hanging in a room with a full view of the light, started activity almost simultaneously with the beginning of darkness. By the same reasoning, a much earlier start of sampling at the Wendelsheim colony after the darkening of the loft should be expected. This was not the case, possibly due to inadequate darkening.

The ecological usefulness of this non-24-hour, endogenous rhythmicity has been touched upon in the course of the preceding discussion. The disadvantages of a purely exogenous regulation of activity are particularly pronounced in the case of bats, but are probably pertinent to all cave- and hole-dwelling species as well. Bats are often torpid during much of the daytime inactive period, appearing uncoordinated and unresponsive to auditory, tactile or visual stimuli. Even if awake and alert they are not exposed to the environment when they roost in unlighted retreats. At the other extreme, the question arises why animals did not develop a purely endogenous system. The majority depend upon both exogenous and endogenous regulators. The endogenous entity may act as a wake-up timer to insure arousal of the bat and the regaining of its full sensory capacities before it tries to perceive its environment. It is perhaps easier in terms of selection to evolve a simple, rather crude timer, capable of correction by the external environment, than to produce one complex enough to predict and account for daily and seasonal changes of the solar day-night cycle.

Conclusions and Summary

Bats living under laboratory or field conditions manifested precise nocturnal activity rhythms. Light-sampling at the light-to-dark transition was apparently the chief means of synchronizing an endogenous, non-24-hour activity rhythm to the daily light cycle.

1. Two horseshoe bats, free-living in separate, small recording rooms, readily adjusted the time of activity to correspond to an L:D schedule.
2. Three horseshoe bats, roosting in a darkened tunnel with no direct view of the 12L:12D schedule of the outside room, anticipated the light-dark change by flying out regularly during the hour before the lights were turned out, then were active for about 8 hours.
3. A colony of *Myotis myotis* exited from a church loft at an average light intensity of 0.05 lux and returned at dawn. Attempted darkening of the church did not result in earlier light-sampling. Illumination of the flight path at the normal exit time resulted in intense sampling but prevented the actual departure of most of the bats.

LITERATURE CITED

Allison, V. C., 1937. Evening bat flight from Carlsbad Caverns. *J. Mammal.*, **18**: 80–82.

Aschoff, J., 1958. Tierische Periodik unter dem Einfluss von Zeitgebern. *Zeitschr. f. Tierpsychol.*, **15**: 1–28.

Aschoff, J., 1960. Exogenous and endogenous components in circadian rhythms. *Cold Spring Harbor Symp. Quant. Biol.*, **25**: 11–28.

Aschoff, J., 1963. Comparative physiology: diurnal rhythms. *Ann. Rev. Physiol.*, **25**: 581–600.

Bruce, V. G., 1960. Environmental entrainment of circadian rhythms. *Cold Spring Harbor Symp. Quant. Biol.*, **25**: 29–48.

Bünning, E., 1963. Die physiologische Uhr. Springer Verlag, Berlin; 153 pp.

Church, H. F., 1957. The times of emergence of the pipestrelle. *Proc. Zool. Soc. London*, **128**: 600–602.

DeCoursey, P. J., 1960. Phase control of activity in a rodent. *Cold Spring Harbor Symp. Quant. Biol.*, **25**: 49–55.

DeCoursey, P. J., 1961. Effect of light on the circadian activity rhythm of the flying squirrel, *Glaucomys volans*. *Zeitschr. f. vergl. Physiol.*, **44**: 331–354.

DeCoursey, P. J., 1963. Function of the light response curve in hamsters. *J. Cell. Comp. Physiol.*, (in press).

Eisentraut, M., 1952. Beobachtung über Jagdroute und Flugbeginn bei Fledermäusen. *Bonner. Zool. Beit.*, **3**: 211–220.

Griffin, D. R., 1958. Listening in the Dark. Yale University Press, New Haven; 413 pp.

Griffin, D. R., and J. H. Welsh, 1937. Activity rhythms in bats under constant external conditions. *J. Mammal.*, **18**: 337–342.

Hodgson, F. S., 1955. An ecological study of the behavior of the leaf cutting ant *Atta cephalotes*. *Ecology*, **36**: 293–304.

Johnson, M. S., 1939. Effect of continuous light on periodic spontaneous activity of white-footed mice (*Peromyscus*). *J. Exp. Zool.*, **82**: 315–328.

Justice, K. E., 1960. Nocturnalism in three species of desert rodents. Ph.D. Thesis, University of Arizona.

Kowalski, K., 1955. The daily rhythm of activity in the mouse-eared bat (*Myotis myotis* Borkh.). *Folia biol. Warszawa*, **3**: 55–64.

Krzanowski, A., 1959. Behavior of bats during the total solar eclipse in Poland on June 30th, 1954. *Polska Akademia Nauk, Acta Theriologica*, **2**: 281–283.

Menaker, M., 1961. The free running period of the bat clock; seasonal variations at low body temperature. *J. Cell. Comp. Physiol.*, **57**: 81–86.

PITTENDRIGH, C. S., 1960. Circadian rhythms and the circadian organization of living systems. *Cold Spring Harbor Symp. Quant. Biol.,* **25**: 159–184.

POHL, H., 1961. Temperaturregulation und Tagesperiodik des Stoffwechsels bei Winterschläfern. (Untersuchungen an *Myotis myotis* Borkh., *Glis glis* L. und *Mesocricetus auratus* Waterh.) *Zeitschr. f. vergl. Physiol.,* **45**: 109–153.

RAWSON, K. S., 1959. Experimental modification of mammalian endogenous activity rhythms. *In:* Photoperiodism and Related Phenomena in Plants and Animals, Edit. by H. Withrow. A. A. A. S., Washington, D. C.

RAWSON, K. S., 1960. Effects of tissue temperature upon diurnal rhythms. *Cold Spring Harbor Symp. Quant. Biol.,* **25**: 105–113.

ROBERTS, S. K., 1960. Circadian activity rhythms in cockroaches. I. The free-running rhythm in steady state. *J. Cell. Comp. Physiol.,* **55**: 99–110.

ROBERTS, S. K., 1962. Circadian rhythms in cockroaches. II. Entrainment and phase shifting. *J. Cell. Comp. Physiol.,* **59**: 175–186.

STEWART, M. C., 1962. Preliminary investigation of circadian rhythms in the pocket mouse *Perognathus intermedius.* M.S. Thesis, University of Wisconsin.

TWENTE, J. W., JR., 1955. Some aspects of habitat selection and other behavior of cavern dwelling bats. *Ecology,* **36**: 706–732.

VENABLES, L. S., 1953. Observations at a pipistrelle bat roost. *J. An. Ecol.,* **12**: 19–26.

BURROWING BEHAVIOR OF DENTALIUM

P. DINAMANI

Oceanographic Laboratory, University of Kerala, Ernakulam 6, South India

In a recent paper on the habits of *Dentalium entalis,* Morton (1959) has remarked that apart from a paper by Yonge (1937) on the ciliary currents in the mantle cavity, little is known about the mode of life of the Scaphopoda. Morton's account deals mainly with the digestive organs, but he has also described feeding and burrowing habits in these animals. In an earlier paper on *D. conspicuum* (Dinamani, 1963), I have pointed out that the mode of feeding by means of the captacula is quite different from what has been suggested by Morton (1959). His account of burrowing deals with the part played by the foot in actual embedment of the animal in the substratum. However, observations on living material of *D. conspicuum* show that activities similar to burrowing are carried on by the animal even after embedment. This paper deals with some of these aspects of burrowing and indicates their possible significance to the feeding habits of the animal.

Living specimens of *Dentalium (Dentalium) conspicuum* Melvill were caught from 30 m. depth off Cochin, and kept in glass tanks filled partly with bottom mud, of sea water in the laboratory.

BURROWING

First stage

In whatever position the animal initially is, during burrowing it has a righting reflex which, when the animal assumes the normal position of rest, brings its dorsal (concave) side upwards (Morton, 1959). This could be clearly demonstrated when the animal is placed in a horizontal position on the surface of the mud with the ventral (convex) side facing away from the substratum. The animal plunges into the substrate in the same position, with the foot proceeding to dig almost at right angles to the body, and when the anterior one-third of the shell has penetrated, the animal goes through a slow twisting motion which brings the concave side up. While the animal performs this twist, it has penetrated further into the mud so that at the end of the twist nearly the whole animal has become embedded. The whole maneuver can be vividly pictured and recorded if a quantity of very fine mud is allowed to settle over the layer of bottom material in the tank before introducing the animal. Now, an animal placed in the tank in the above position leaves a clear trail on the surface of the mud (Fig. 1A). The apex of the shell describes an arc and makes a half turn as the animal twists through nearly 180°, and the direction of the twist and its extent are marked by the apex of the shell on the fine mud (Fig. 1 A and D, *t'*).

Second stage

While "reading" the trails left on the mud surface by a few specimens, another feature of burrowing became noticeable. In addition to the trail left by the body of the animal as it drags forward and digs into the substratum (*t* in Fig. 1 A) and at a distance nearly equal to its own length in front, a saucer-shaped area of loose sediment was observed (*t″*, Fig. 1 A). At first the significance of these markings was not understood, but it was being regularly observed wherever a *Dentalium* had burrowed. In order to verify whether the burrowing habits of the animal were the cause of these formations, a specimen was kept under close observation after its passage from the surface into the mud. Soon after it had embedded itself in the normal position, the surface of the mud showed faint hair-line cracks at a site approximately above the buried front part of the animal. These cracks widened slowly as mud particles around became displaced, and eventually a slight depression appeared on the surface, clearly indicating that the burrowing activity of the animal had brought on this effect. Animals introduced in glass troughs filled partly with coarse sand or gravel do not leave a clear trail, but if a layer of fine mud is first allowed to settle on the surface of such material, the burrowing activities become clearly evident.

The action of the foot was later closely followed in a number of animals placed close to the walls of the glass trough, where their activity was partially visible through the glass partition. After the animal embeds itself, the pointed tip of the foot slides into the substratum (Fig. 1 B) in a series of wave-like movements, and at the same time the whole foot is moved in an arc towards the animal. This has a raking action on the sediment, and the pedal lobes, which are held pressed against the sides of the foot during these movements, are now erected and swung back (Fig. 1 C). Particles of mud loosened by the foot in front are shovelled back by the pedal lobes, and soon a small space is cleared around the foot. During these movements mud particles trickle down into the space cleared by the foot, and hair-line cracks appear on the surface of the mud above. The animal shifts its position occasionally, partly drawing back or going forward, with the result that when a space has been cleared around the front end of the animal, the column of mud immediately above the foot has developed many cracks and interstices, and as a result becomes loose-textured. Water also trickles through the cracks into the area cleared by the animal, and the pedal lobes acting in this fluid medium have a kind of churning action on the sediment, and occasionally a small quantity of water and mud is forced out through the cracks. This action repeated many times results in a small depression at the surface, formed of loose sediment (Fig. 1 A and D, *t″*). Periodically the foot is fully retracted into the mantle cavity, and this takes place in a definite sequence. First, the tip of the foot contracts, while the pedal lobes are fully expanded and held closely appressed to the mantle fringe behind. Now, the mantle contracts around the foot in the form of a collar till the pedal lobes and the tip of the foot are withdrawn into the mantle cavity. This behavior has two advantages: no water or foreign matter is allowed to enter the mantle cavity, and the particles of mud adhering to the foot and the pedal lobes are also wiped free by the mantle fringe.

DISCUSSION

Action similar to burrowing on the part of *Dentalium* after it has embedded itself in the substratum results in a loosening of the sediment in an area immediately in front of the animal. As the sediment is worked loose by the foot, cracks develop

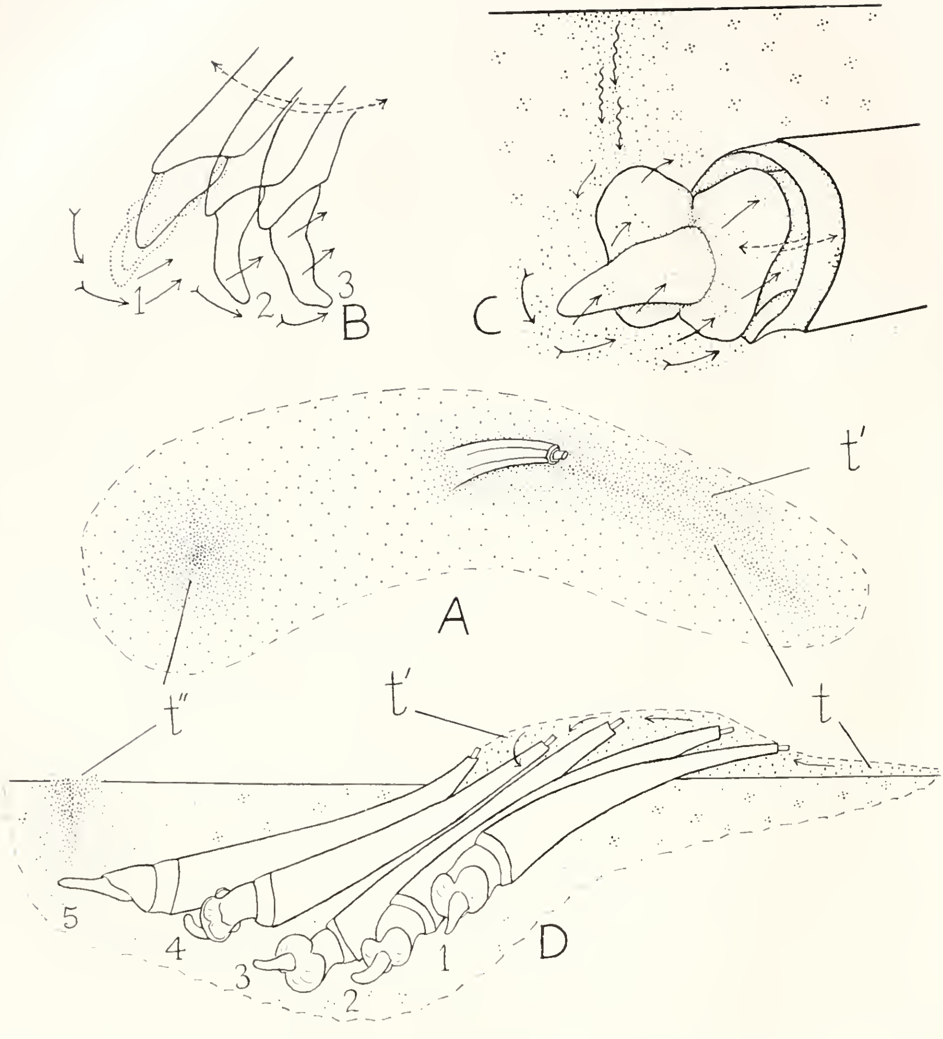


FIGURE 1. *Dentalium (Dentalium) conspicuum* Melvill. A. Markings made by the animal on the surface of the sediment as it drags itself forward (*t*), twists over (*t′*) and embeds itself; *t″* shows the loosened area of sediment above the front part of the animal. B. Movements of the foot as it slides into the substratum (shown by dotted outline) and is swung through an arc (indicated by dotted arrows). C. Wafting movements of the pedal lobes (indicated by dotted arrows) and the resulting shifting of sediment. D. Lateral view showing successive stages of the twisting motion (direction of twist indicated by arrows) which brings the concave dorsal side to the top at the end of burrowing, and the communication with the surface established through loosened sediment (*t″*) by the front part of the animal. In B and C, feathered arrows indicate direction of movement of foot and pedal lobes against the substratum, while unfeathered arrows indicate movement of sediment particles. In B and D, the sequence of events is indicated serially as 1, 2, 3. . . .

in the column of sediment lying immediately above and, aided by the continued wafting action of the pedal lobes, mud particles become displaced and begin to sink in from above. The net result is that a communication is soon established between the front end of the animal and the water column above through the intermediary of cracks and passages of the overlying sediment (Fig. 1 D). Water and sediment particles could then trickle down from above through the loosely-textured layer or be driven out due to the impelling action of the pedal lobes. It is suggested that this behavior of the animal may have an important functional basis: the loosening of the substratum by the foot and the pedal lobes results in a churning of the sediment that may help the animal to get at food particles among them with the captacula, the animal being entirely a deposit feeder. The sifting action becomes particularly important in capturing Foraminifera which occur in the deposit. These are generally considered to be the chief article of diet of *Dentalium* (Morton, 1959). The communication established through the overlying column of sediment may also help in drawing in particles of detritus from the surface. The stomach contents of the present species showed diatoms, single algal cells and unidentifiable detritus, and this could be available in quantities only from the surface deposits. It is also obvious that the captacula can seek for food only in a partly fluid medium, particularly since feeding is carried on through a ciliary tract in the captaculum (Dinamani, 1963). In those species living on sandy or gravelly bottoms, the interstices naturally occurring between particles of sediment may be sufficient to "ventilate" the stratum at the anterior end of the animal, though here, also, the burrowing action of the foot should have some effect.

The general view that connection between the scaphopod and the water column exists only through the posterior mantle tube can be held tenable if we take into consideration only the current of water going in and out of the mantle cavity for purposes of respiration, rejection of waste and extrusion of sexual products. This would fail to account for the important function of feeding, since the animal is a detritus feeder, feeding by means of the captacula. The captacula could obviously seek through the sediment for food only if the sediment is partially loosened. This is borne out by the fact that only after a period of digging among the sediments and the withdrawal of the foot do the captacula emerge for feeding (Morton, 1959; Dinamani, 1963). The behavior of the animal in establishing a communication with the exterior at the forward end may be for drawing in detritus matter from the surface deposits, and may therefore have an important bearing on nutrition. Periods of feeding and burrowing activities appear to succeed each other, and it would seem as if the animal's main burrowing activities are directed towards digging for food in the substratum.

Egg laying

On March 20, 1963, one of the specimens, measuring 65 mm. in length, soon after introduction into a larger trough of fresh sea water, laid a batch of eggs. The specimen, placed on the surface of the mud in an upside down position (with the ventral side up), remained at the surface for over 15 minutes. A small cloud was then observed issuing from the posterior aperture, being steadily impelled from the mantle cavity. It was deposited on the surface of the mud in the form of a long ribbon 6–7 cm. long. The ribbon consisted of small groups of 4 or 5 eggs each, barely touching each other, and a superficial count showed nearly 1000 eggs. The eggs were of a faint pinkish hue and measured 0.17 to 0.18 mm. in diameter, and had a clear membrane. The eggs were collected and introduced into a series of fingerbowls with fresh sea water, but because of high mortality, stages of development could not be followed closely.

SUMMARY

Observations on living material of *Dentalium* (*Dentalium*) *conspicuum* Melvill show that after the animals have embedded themselves in the substratum, burrowing activities of the foot are directed towards loosening an area of the substratum around the foot. This causes sinking and displacement of material in the column of sediment overlying the foot, resulting in cracks and minute passages through which connection between the animal and the surface is established. The action of the foot and the pedal lobes in loosening and sifting sediment materials may enable the captacula to seek among them for food, such as Foraminifera and detritus. The connection established with the surface may also help in drawing in more detritus matter from the surface. The main digging activities of the animal appear to be directed towards seeking for food in the sediment. A note is also added on the mode of egg-laying observed in a single specimen.

LITERATURE CITED

DINAMANI, P., 1963. Feeding in *Dentalium*. *Proc. Malacol. Soc. London,* **35**: (in press).

MORTON, J. E., 1959. The habits and feeding organs of *Dentalium entalis*. *J. Mar. Biol. Assoc.,* **38**: 225–238.

YONGE, C. M., 1937. The circulation of water in the mantle cavity of *Dentalium*. *Proc. Malacol. Soc. London,* **22**: 333–338.

NUTRIENT TRANSPORT IN STARFISH. I. PROPERTIES OF THE COELOMIC FLUID[1]

JOHN CARRUTHERS FERGUSON

Department of Zoology, Cornell University,[2] Ithaca, New York

The means by which starfish transport nutrient materials from absorptive sites, in the digestive glands, to the various other tissues has been the subject of much uncertainty. At least five distinct mechanisms have been proposed by which this might be accomplished. They are by (1) circulation through the perivisceral coelom; (2) transport *via* the coelomocytes; (3) movement through the haemal channels; (4) circulation through the perihaemal spaces; and (5) circulation through the water vascular system. Little evidence has appeared to support any of these theories, and arguments may be directed against each of them.

The most obvious route for nutrient transport is through the pervisceral coelom. This is a spacious cavity, whose fluid accounts for 23–27% of the body weight in *Pisaster ochraceus* (Feder, 1956). It contains or lies adjacent to most of the important structures of the body. Early investigations, particularly of the enzymes present, led Cuénot (1887a, 1887b), Chapeaux (1893), and Cohnheim (1901) all to propose that digestion of food products was completed in the coelom by the action of the coelomocytes. Absorption of food products from the chamber was demonstrated by Van der Heyde (1922, 1923a), who observed that relatively high concentrations of nutrients injected into the body cavity of *Asterias* would rapidly disappear. Irving (1924) and Budington (1942) described directly ciliary currents in the coelom of starfish and concluded that these must function to distribute dissolved nutrients.

Several arguments may be directed against the hypothesis of coelomic nutrient transport. First, certain structures, such as the ectoneural nervous system and many muscles, are completely inaccessible to the perivisceral coelomic fluid and thus could not receive nutrients from it. Second, the currents in this fluid might well function for the distribution of respiratory gases without being significant in nutrient transport at all. Third, and most important, reported analyses of the coelomic fluids of asteroids show that their compositions, except for the coelomocytes, are almost identical to that of sea water, and that they contain only very small amounts of organic materials (Meyers, 1920; Delaunay, 1931; Giordano *et al.,* 1950; and Greenfield *et al.,* 1958). Non-protein nitrogen is reported to occur at a concentration of 8 to 44 μg. per ml. of fluid. Protein is difficult to measure but may be present to the extent of a few μg. per ml. Such values are far less than the corresponding concentrations of substances in the circulatory fluids of most other animals (*e.g.*, non-protein nitrogen in human blood = 250 to 400 μg. per ml. according to White *et al.*, 1954).

---

[1] This work represents a part of a dissertation submitted to Cornell University in partial fulfillment of the requirements for the Ph.D. degree, granted in June, 1963. The author is greatly indebted to Dr. John M. Anderson for his interest in this project, and for his advice and encouragement. Much of the research was performed during tenure of a NSF Summer Fellowship. Additional financial support was provided from NSF Grant No. G-20744 to Cornell University.

[2] Present address: Florida Presbyterian College, St. Petersburg, Florida.

The second possible mechanism of nutrient transport is the coelomocytes, which are ubiquitous throughout the body. There are several types of these cells in starfish (Boolootian and Giese, 1958; Boolootian, 1962) which appear to have different functions. They were first suspected of transport after being observed to accumulate dyes and fatty materials fed to the animals (Cuénot, 1887b; Chapeaux, 1893; Cohnheim, 1901). Further support for this view was offered by Van der Heyde (1922, 1923b), who showed that *in vitro* they could absorb large quantities of glucose very rapidly from coelomic fluid. Many workers have pointed out, however, that phagocytic activity is a natural method of excretion in these forms. Most of the reported experiments fail to distinguish between such elimination and true nutrient transport. Conclusive evidence that coelomocytes play a significant part in nutrient translocation, even for only special classes of compounds such as lipids, has never been presented.

The three other systems (haemal, perihaemal, and water vascular), often considered significant in transporting nutrients in starfish, have received practically no experimental attention. For the present, their function in this regard must be considered purely speculative.

The answers to the problems of how nutrients are transported about the bodies of asteroids have remained unknown mainly because suitable methods for their elucidation have not existed. In the past few years new analytical techniques have become available involving radioactive tracers and extremely sensitive microchemical analyses. The present investigation was undertaken in an effort to use some of these to determine the basic properties of the coelomic system in regard to the handling of nutrients. Three principal approaches were used. First, analyses were made to measure the concentrations of possible nutrient substances in serial samples of coelomic fluid taken from animals at various times after feeding or during fasting. Second, measurements were made of the concentrations of several labeled nutrients in samples of coelomic fluid and coelomocytes drawn at periodic intervals following the feeding of these substances to starfish. The ultimate distribution of the tracers in various body parts was also determined. And third, labeled nutrients were injected directly into the coelomic cavities of starfish so that their subsequent distribution could be followed by serial analyses of the coelomic fluid and terminal tissue samples.

Materials and Methods

All the animals used were freshly collected, active specimens of *Asterias forbesi* from the Woods Hole region, weighing about 150 grams. They were kept in the laboratory in wooden and glass tanks supplied with adequate quantities of fresh, well-aerated sea water at a temperature of 20° to 23° C. These specimens were fasted from one to several weeks and then fed 1- to 2-gm. portions of clam (*Mercenaria mercenaria*) before periodic sampling of their coelomic fluids was begun to measure the concentrations of dissolved nutrients. In other experiments, tracers were administered in food by injecting about 10 microcuries (μc.) of the labeled nutrients into the visceral mass of small specimens of *Spisula solidissima* from which a small piece of one valve, but not the mantle, had been removed, and feeding the whole clam to the starfish. Some of the tracer was undoubtedly lost into the sea water during the initial stages of feeding, making it impossible to determine the exact dose ingested. In another set of experiments, designed to measure the absorption of nutrients from the coelomic cavity, starfish were injected in the middle of one ray with about 5 μc. of NaOH-neutralized tracer made up to 0.5 ml. in sea water. Samples of fluid were drawn from an opposite ray to avoid direct contamination with the site of injection.

All the tracers employed were C^{14}-labeled products isolated from the alga, *Chlorella pyrenoidosa*, and supplied by the New England Nuclear Corporation of Boston, Mass. The following reagents were used: (1) *Algal protein-*C^{14} *hydrolysate*—0.10 millicurie (mc.) (0.124 mg.) per ml. in 1.0 N HCl. (2) *Glycine*-1-C^{14}—0.10 mc. (0.314 mg.) per ml. in 0.01 N HCl. (3) *D-glucose-*C^{14} (U.L.)—0.10 mc. (7.4 mg.) placed in 1 ml. H_2O. (4) *Palmitic*-1-C^{14} *acid*—0.10 mc. (3.5 mg.) placed in 0.5 ml. peanut oil.

Sampling was achieved by withdrawing coelomic fluid from near the tip of a ray with a syringe fitted with a 23-gauge Huber point needle. For the chemical analyses, 1.5-ml. samples of fluid were taken and placed in small vials containing a few pieces of shredded filter paper. After clotting had taken place, the contents were decanted into 15-ml. centrifuge tubes and spun down for ten minutes at 4300 rpm. The supernatant was then decanted through Whatman No. 1 filter paper and used for duplicate analyses.

Total nitrogen was determined by digesting a 0.5-ml. aliquot of the filtered fluid with sulfuric acid and $CuSeO_4$ catalyst in a 10-ml. Erlenmeyer flask. The ammonia released by the digestion was diffused onto a glass rod placed in the flask and washed off into 5 ml. of ammonia-free water. A 2-ml. aliquot was then placed into a cuvette, together with 1 ml. of 0.5% sodium nitroprusside solution, 1 ml. of 2% phenol solution, and 1 ml. of 1:20 sodium hypochlorite in 0.5 N NaOH. The color was developed by heating the cuvette 10 minutes in hot water, and read against a blank at 640 mμ on a colorimeter. Free ammonia was measured in the same manner, omitting the digestion step and using a different dilution to give the samples the best density for reading. Attempts were made to measure non-protein nitrogen by first precipitating the protein with TCA. As the concentrations involved were very low, it is doubted that this procedure was adequate for the purpose. The nitrogen values of a few tissue samples were determined by a standard Kjeldahl technique.

Reducing sugar was measured by placing 0.5-ml. aliquots of the filtered fluid in test tubes, precipitating the protein with the BaOH–$ZnSO_4$ method of Somogyi (1942), removing the precipitate with a fine-porosity alundum crucible, and developing color in the filtrate by the alkaline-ferricyanate method of Park and Johnson (1949), slightly modified with respect to reagent concentrations. Some inhibition of the color development by the ionic composition of the samples was experienced, but this was not sufficient to destroy the usefulness of the method. The samples were read against sea water blanks and compared against fresh standard solutions of glucose made up in sea water.

In the tracer studies, 0.5 ml. of coelomic fluid was taken and placed directly into

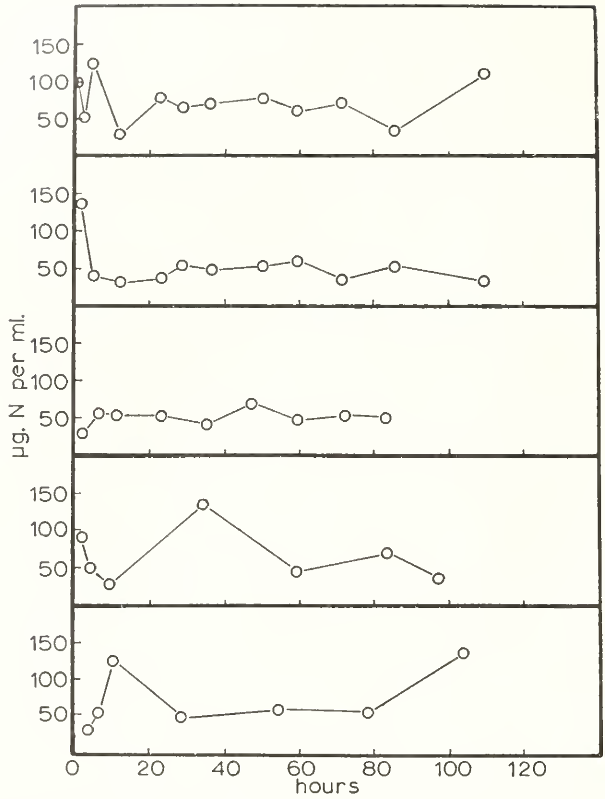


FIGURE 1. The concentrations of total nitrogen observed in the cell-free coelomic fluid of five animals at various times after feeding.

a 15-ml. centrifuge tube. After centrifugation, 0.25 ml. of supernatant was mixed with 0.5 ml. of distilled water and plated directly (in duplicate) as 0.25-ml. aliquots onto stainless steel planchets. The pellet, containing the coelomocytes, was rinsed, dissolved in 0.5 ml. of concentrated HCl, diluted to 1 ml. and plated (in triplicate) as 0.25-ml. aliquots onto stainless steel planchets. At the conclusion of these experiments the digestive glands and pieces of the oral and aboral body wall were removed, weighed, dissolved in hot nitric acid, and diluted to a volume of 25 ml. with distilled water or an acetone-distilled water mixture. Triplicate 0.5-ml. aliquots of these solutions were plated onto corrugated stainless steel planchets. All the planchets were dried under a heat lamp and counted in a Nuclear Chicago shielded, low-background counter fitted with an automatic sample changer and a thin "Micromil" window. This machine was indispensable for evaluating many of the low-activity samples encountered. To facilitate comparisons of the different elements examined, the fluid and coelomocyte counts were converted to the common units of millimicrocuries (mμc.) of activity per ml. of coelomic fluid by multiplying them by factors determined from counts made on known standards. The counts of the tissue specimens were corrected for self-absorption by comparison with previously calculated absorption curves, and similarly converted to units of mμc. per gm. wet-weight of tissue.

To measure the effect of the repeated sampling of the serially analyzed specimens, large amounts of fluid were removed from two animals after they had been carefully weighed. These animals were then reweighed at frequent intervals for several days, so that the rate of replacement of the fluid might be estimated.

RESULTS

Total nitrogen

The concentrations of total nitrogen maintained in the coelomic fluids of five animals after feeding are shown in Figure 1. Four fasted control animals produced similar records. Analysis of the data by taking running averages failed to show any significant differences between the two groups, even though there appeared to be a suggestion of a slight initial peak in the mean values of the fed animals during the first hour or so after feeding. The mean value obtained for all the samples was 63.5 μg. N per ml. of coelomic fluid, but all the specimens demonstrated marked and apparently random fluctuations from the mean value.

Free ammonia

The concentrations of free ammonia maintained in the coelomic fluids of three animals after feeding and two fasted animals are presented in Figure 2. The mean

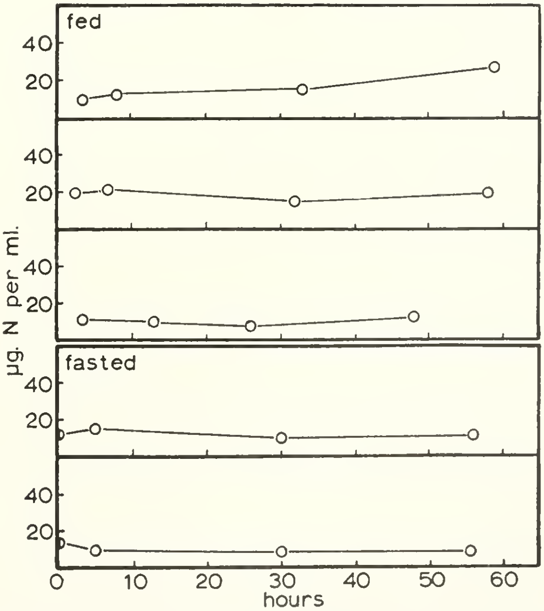


FIGURE 2. The concentrations of free ammonia nitrogen in the coelomic fluids of three fed and two fasted animals.

value for all the analyses was 12.8 μg. NH_3 N per ml. of fluid. There was no significant difference between the two groups. The mean value for the first 50 hours after feeding was 13.3 ± 1.31 (± S.E.) while the comparable value for the fasted specimens was 10.9 ± 0.96. "*P*" in this case was greater than 0.20. It may be noted that the ammonia levels remained much more stable than the total nitrogen concentrations.

Non-protein nitrogen

Because of the inadequacy of the method, only two animals were analyzed for non-protein nitrogen. These had values of 30 and 48 μg. per ml. As total nitrogen in these specimens was 41 and 63 μg. per ml., protein nitrogen must have represented 27% and 24% of the total present.

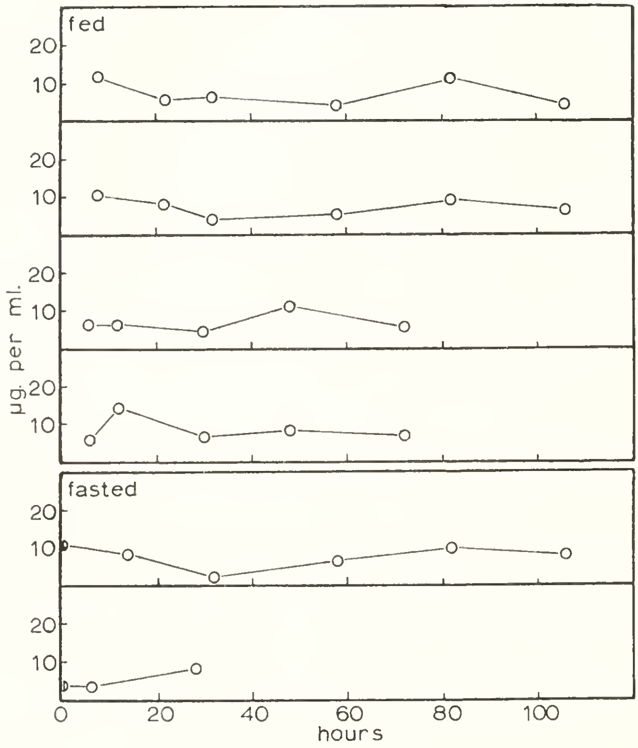


FIGURE 3. The levels of reducing sugar, measured as glucose, observed in the coelomic fluid of four animals following feeding and two fasted animals.

Reducing sugar

The concentrations of reducing sugar, measured as glucose, found in the coelomic fluids of four fed and two fasted animals are shown in Figure 3. The quantities encountered were extremely small. The mean value for all the determinations was 6.9 μg. of glucose per ml. of coelomic fluid. There was no significant difference between the fed and the fasted specimens in the mean concentrations maintained over the first 50 hours ("*P*" greater than 0.30). The former mean was 7.96 ± 1.10

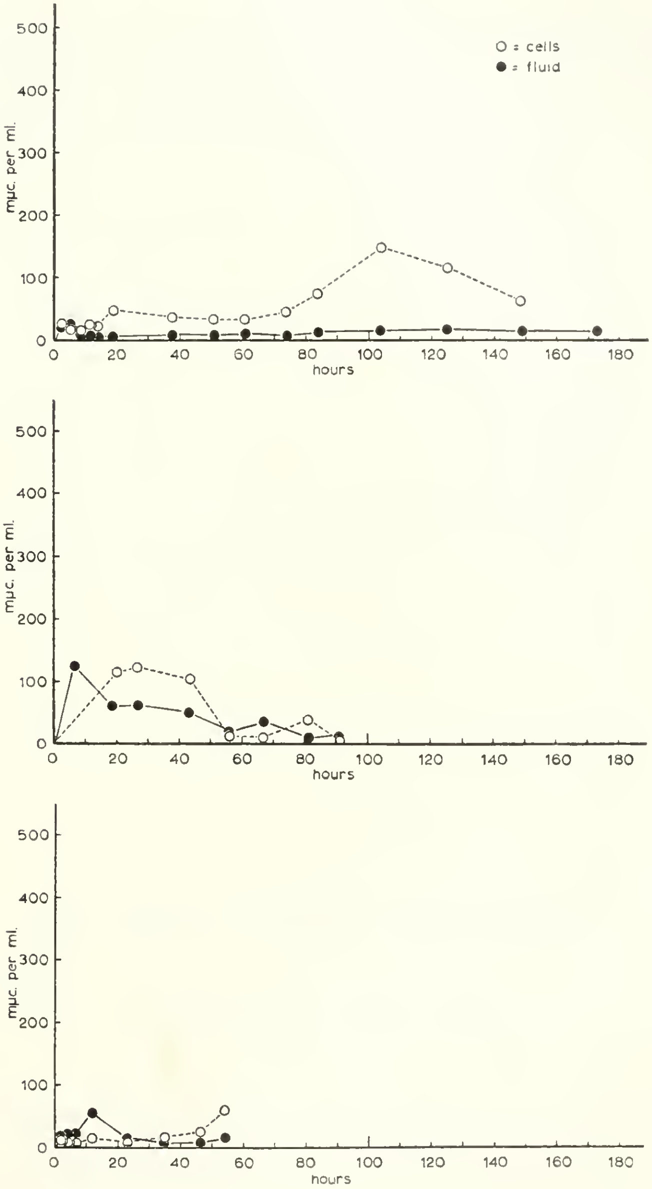


FIGURE 4. The levels of radioactivity that occurred in the cell-free coelomic fluid (fluid) and in the coelomocytes (cells) of three specimens after they were feed 5 to 10 μc. of algal protein-C^{14} hydrolysate.

(± S. E.); the latter, 5.97 ± 1.30. Individual values showed only moderate fluctuations from the mean.

Ingested C^{14}-labeled nutrients

Many similarities were seen among the animals fed the different labeled nutrients. The distribution of activity in the three specimens fed algal protein-C^{14} hydrolysate is shown in Figure 6. The amounts of activity in the fluid portions and the coelomocytes always remained very low compared to the total dose fed. In the specimens fed labeled algal protein hydrolysate and glucose, there was some tendency for a high initial peak in the activity of the fluid and for a gradual leveling-off at a lower concentration. The coelomocytes generally showed a tendency to increase in activity throughout most of the experiment. In the specimens fed palmitic-C^{14} acid, the peak in fluid activity appeared later than that seen with the other nutrients, and one of the two animals studied showed almost no activity present in the fluid after the first day. Similarly, the gradual increase in coelomocyte activity did not seem to occur in these individuals.

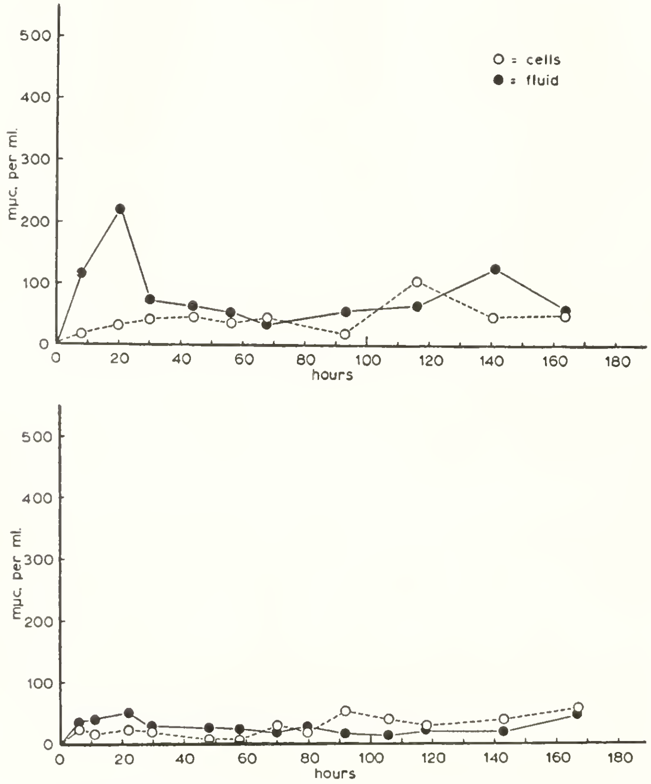


FIGURE 5. The appearance of radioactivity in the coelomic fluid components of two animals which had been fed 5 to 10 μc. of glucose-C^{14}.

The final distributions of activity in the fed animals are compared in Table I. It is apparent that most of the nutrient ingested was retained in the digestive glands, even a week after feeding, and that comparatively low levels persisted in the fluid and coelomocyte fractions. This is especially true of the specimens fed palmitic-C^{14} acid. Significant portions of the activity were found in samples of both the oral and aboral body walls.

The distribution of activity with respect to the organic content of the different tissues is shown in Table II where the values presented in the preceding table have been expressed in terms of the activity per mg. of average nitrogen content for the various elements. While differences still exist in the distribution, the values have tended to approach one another. This is especially true for the fluid, coelomocyte, and digestive gland components.

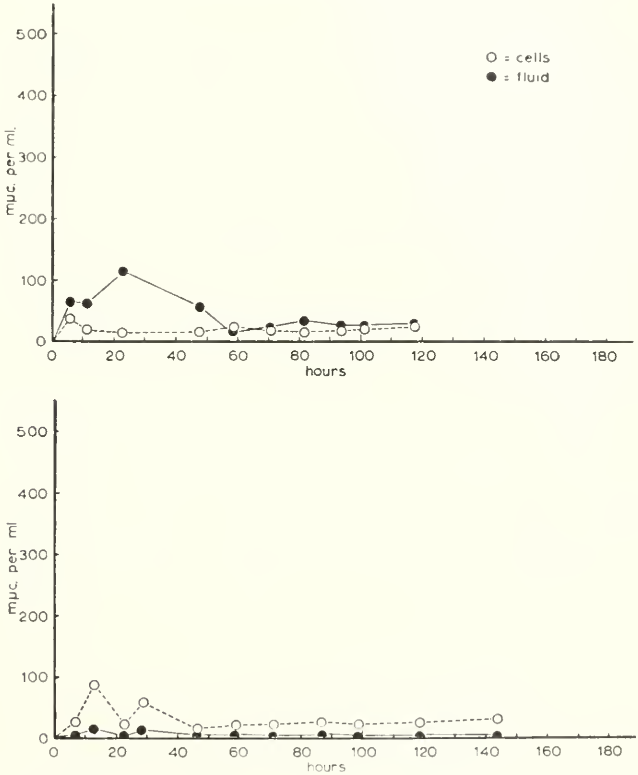


FIGURE 6. The levels of radioactivity found in the coelomic fluid components of two animals that had been fed palmitic-C^{14} acid.

Injected C^{14}-labeled nutrients

The activities found in the components of the coelomic fluid of the three specimens injected with algal protein-C^{14} hydrolysate are given in Figure 7. Those

TABLE I

The final distribution of radioactivity at the time of sacrifice in animals which had been fed C^{14}-labeled nutrients

Nutrient fed	Time, hours	Coelomic fluid		Digestive gland mμc./gm.	Aboral body wall mμc./gm.	Oral body wall mμc./gm.
		Fluid mμc./ml.	Cells mμc./ml.			
APH	54	1.5	5.9	400	36	31
APH	91	0.8	0.4(?)	340	20	16
APH	173	1.8	5.9	405	—	63
Glucose	164	5.6	4.9	3300	140	385
Glucose	166	5.0	5.4	2450	110	460
Palmitic acid	118	2.8	2.6	600	370	335
Palmitic acid	144	0.1	0.3	1005	10	20

for the two glycine-C^{14}-injected animals are presented in Figure 8. The data for the two glucose-C^{14}-injected animals are shown in Figure 9. These experiments show a number of interesting features. First, it is apparent that there is considerable circulation of fluids from one ray to another, as all the samples were withdrawn from a ray opposite the one that had received the injection. Second, the curves clearly indicate that material is removed from the fluid and is taken up, in part, by the coelomocytes. These cells have relatively less affinity for glucose than for the other nutrients.

Table III, showing the final distribution of the tracers in the animals, further confirms these observations and reveals a few additional points. The most important fact is that the digestive glands are the organs that have removed most of the activity from the coelomic fluid, while the other tissues, including the coelomocytes, have also contributed to the process. In some cases the digestive glands in the

TABLE II

The final distribution of radioactivity per mg. of tissue nitrogen in animals that had been fed C^{14}-labeled nutrients

Nutrient fed	Time, hours	Coelomic fluid		Digestive gland mμc.	Aboral body wall mμc.	Oral body Wall mμc.
		Fluid mμc.	Cells mμc.			
APH	54	25	30	13	2	2
APH	91	14	2(?)	10	1	1
APH	173	30	30	12	—	5
Glucose	164	93	25	96	10	29
Glucose	166	83	27	73	8	25
Palmitic acid	118	47	13	18	25	25
Palmitic acid	144	2	1	30	1	2

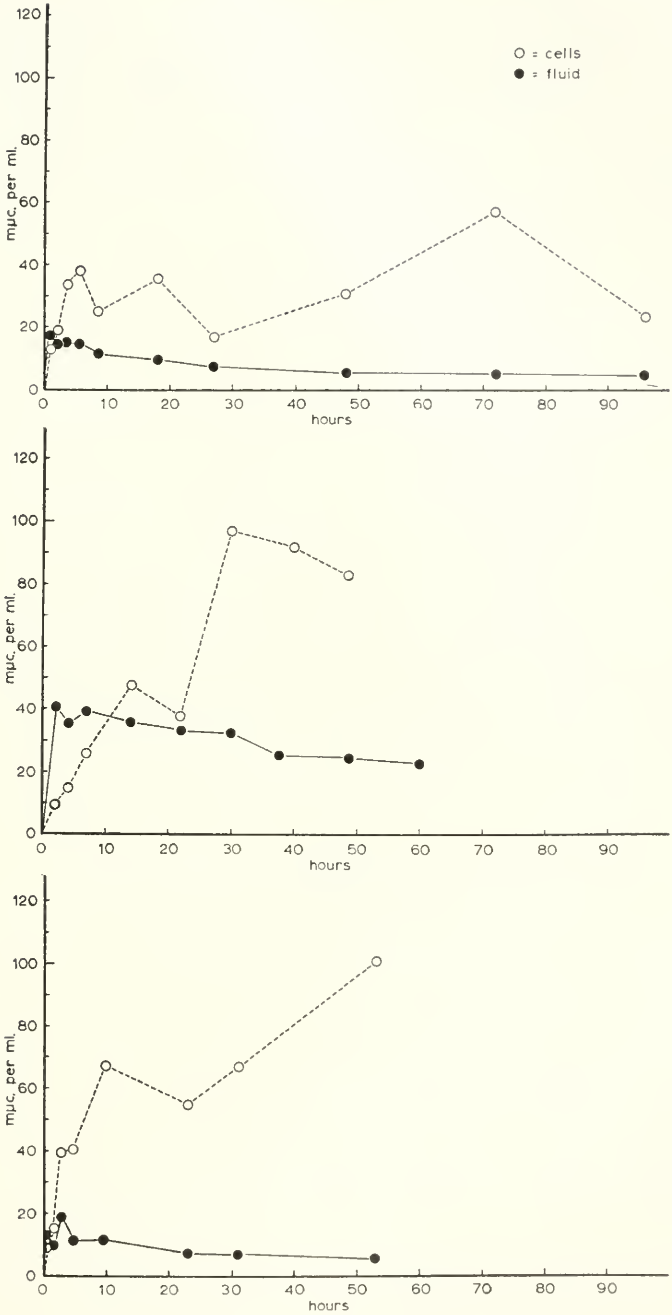


FIGURE 7. The levels of radioactivity found in the coelomic fluid components of three animals following the injection of algal protein-C^{14} hydrolysate into their body cavities. The samples were withdrawn from a ray opposite the one which was injected.

TABLE III

The distribution of radioactivity, at the time of sacrifice, in animals that had been injected with C^{14}-labeled nutrients

Nutrient injected	Time, hours	Coelomic fluid		Digestive gland mμc./gm.	Aboral body wall mμc./gm.	Oral body wall mμc./gm.
		Fluid mμc./ml.	Cells mμc./ml.			
APH	53	5.5	10.0	940	—	—
		21.1*	106.0*	1240*		
APH	96	4.3	23.9	20	—	46
		—	—	1200*	—	150*
Glycine	96	4.1	24.0	90	65	48
Glycine	93	2.2	19.6	270	140	105
Glucose	39	2.1	3.5	530	2	6
		1.8*	13.7*	800*	240*	120*
Glucose	56	1.5	5.5	115	20	38
		1.2*	4.8*	750*	150*	140*

* Injected ray.

injected ray took up most of the activity, while in others, the glands of the uninjected rays absorbed nearly as much, probably reflecting differences in the rates of circulation of fluids between the two areas. In the glucose-C^{14}-injected animals, the marked differences between digestive gland activities and those of the body wall components are probably significant. They suggest that tissues other than the digestive glands may have little capacity to store glucose.

TABLE IV

The final distribution of radioactivity per mg. of tissue nitrogen in animals that had been injected with C^{14}-labeled nutrients

Nutrient injected	Time, hours	Coelomic fluid		Digestive gland mμc.	Aboral body wall mμc.	Oral body wall mμc.
		Fluid mμc.	Cells mμc.			
APH	53	92	506	28	—	—
		340*	530*	37*	—	—
APH	96	72	120	1	—	—
		—	—	36*	—	11*
Glycine	96	68	120	3	4	4
Glycine	96	37	98	8	10	8
Glucose	39	35	18	16	0	0
		30*	69*	24*	17*	9*
Glucose	56	25	28	3	1	3
		22*	24*	22*	10*	11*

* Injected ray.

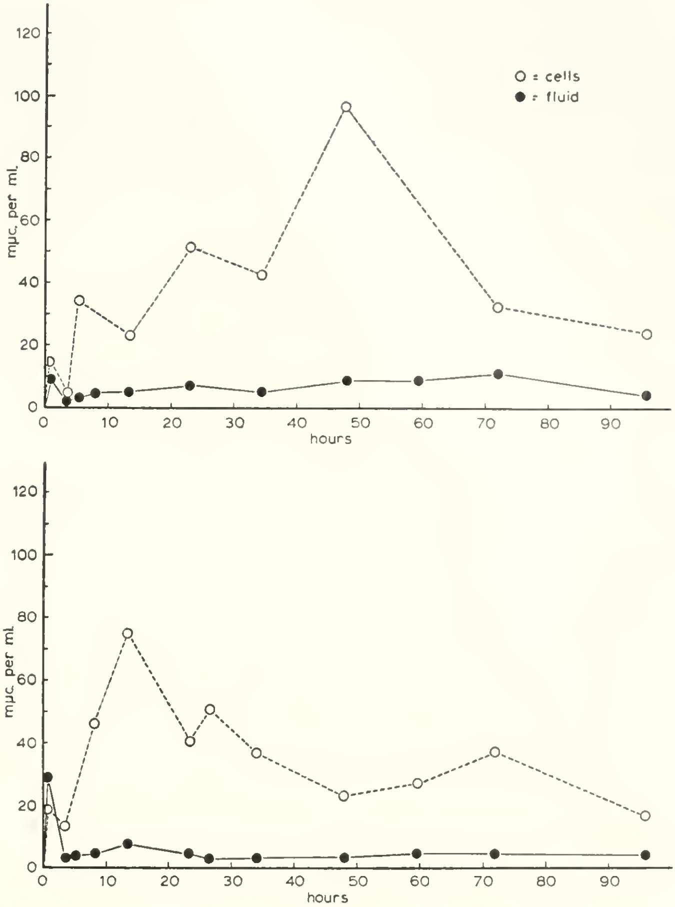


FIGURE 8. The radioactivity in the coelomic fluid components of two animals which had been injected with glycine-C^{14}.

The final distribution of the tracers in the specimens, expressed in terms of the activity per mg. of nitrogen content, is given in Table IV. The data show many of the same properties as were demonstrated in the fed animals, particularly with respect to the partition of activity between the fluid and the digestive glands. The coelomocytes, however, have taken up a somewhat greater proportion of activity as compared to the digestive glands. In the specimens injected with glucose-C^{14}, it appears that all the coelomic fluid is in equilibrium with the activity found in the digestive glands of the injected ray.

Sampling effects and fluid retention

In the experiments measuring the capacity of starfish to replace fluid, there was a slight additional loss of weight, and presumably fluid, after sampling, followed by a gradual regain of weight. A drop in weight was again observed at about the fourth day in both animals, but this may have been due to a slight fluctuation in the salinity. In any case, it is apparent that starfish replace lost body fluids only very slowly, but can survive well and be active with a deficit of fluid volume. In relation to the present experiments these results suggest that the fluids of the sampled animals remained fairly constant in composition and did not become diluted by freshly absorbed sea water coming in to replace the volumes removed.

Discussion

Nitrogen in the coelomic fluid

The data obtained from the nitrogen analyses of the coelomic fluid of the fed and fasted animals show that *A. forbesi* maintains significant amounts of nitrogenous substances in its internal fluids, even though the concentrations of these substances are extremely low. On the average, 20% of the total nitrogen content is present as free ammonia, apparently the principal excretory product of these animals. There is some evidence that nearly 25% of the total nitrogen content occurs as precipitable protein, which could play only a minor role in nutrient transport. Only the remainder, probably not more than 55% of the coelomic fluid nitrogen, could represent compounds of significance as nutrients in transit between the various tissues. Small fractions of this must also represent non-nutritional compounds, such as urea and uric acid, as these are reported to be present in the starfish in low

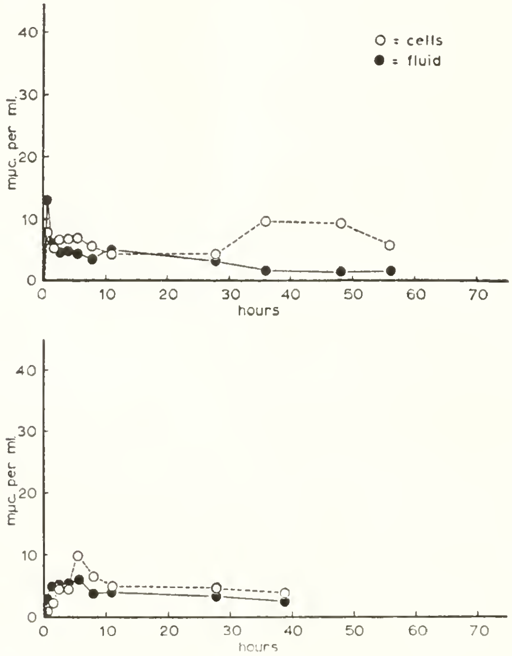


FIGURE 9. The radioactivity in the coelomic fluid components of two animals injected with glucose-C^{14}.

concentrations (Myers, 1920; Delaunay, 1931). But even taking these into account, the data would suggest that there is still a mean concentration of nitrogen in the coelomic fluid of 30 to 35 μg. per ml. This would consist of the amino acids and other low-molecular-weight compounds which one would expect to be most important in the transport process.

It appears, then, that while the total nitrogen content of the coelomic fluid of starfish is very low, the fraction that could represent nutrients in transport is very high—approximately 50%. Comparable values for other animals are uncertain, but from the reported composition of human plasma, nutrients could represent only a fraction of one per cent. Starfish apparently lack all the clotting and immunity proteins present in most other animals, relegating the functions served by these to the coelomocytes. In addition, they have only very low levels of nitrogenous excretory products, no respiratory proteins, and none of the other materials which frequently make up significant fractions of the plasma nitrogen of other animals. Thus, in spite of the low content of nitrogenous substances in their coelomic fluid, starfish still maintain sufficient concentrations of these materials to have significance in nutrient transport.

It is not surprising that no significant differences occur between the nitrogen contents of the coelomic fluids of fed and fasted animals. Studies by Anderson (1952, 1953) and others have indicated that the digestive glands are the principal sites of nutrient storage. There would need, then, be no rapid movement of freshly ingested materials to distant regions of the body. They would simply be retained in these organs until they were needed, and then released.

It may be pointed out that these results are in contradiction to the observations of Van der Heyde (1922), who reported finding fairly large concentrations of amino acid in the coelomic fluid of an *Asterias* when this animal was sampled during feeding. One possible explanation for this discrepancy is that his specimen might have had an injured cardiac stomach, so that when he drew his sample, he may have aspirated some of the digestive material.

The marked fluctuations of the total nitrogen content observed in the coelomic fluid of starfish are not so unusual as they may seem. They can, for instance, be compared with the rather wide range of values reported for the normal amino acid content of human plasma (40 to 80 μg. per ml.). Similarily, Greenfield *et al.* (1958), in their study on several West Coast echinoderms, reported observing variations in the nitrogen content of freshly collected specimens of *Pisaster ochraceus.* They attributed to these to the length of time since the last feeding in the field. In light of the present data, however, the fluctuations more likely reflect the normal variability occurring in individual animals.

It is significant that the free ammonia content of the coelomic fluid remains relatively constant in spite of the variations in the total nitrogen content. Metabolism and excretion must be much steadier processes than those accounting for the presence of the other nitrogenous materials. Also of interest is the hint from the fragmentary data available that the proportion of protein to non-protein nitrogen in the coelomic fluid remains constant regardless of the total nitrogen content. Further experiments to verify the significance of this observation need to be made. If it is true, it would suggest that proteins as well as amino acids are used as metabolites, and the concentrations of both fluctuate with the needs of the animal.

Reducing sugar in the coelomic fluid

The very low levels (6.9 μg. per ml.) of reducing sugar found in the coelomic fluid of starfish are in marked contrast to the concentrations of these substances in the circulatory fluids of other animals. The normal values for the plasma of man depend somewhat on the test used, but generally range from 700 to 1000 μg. per ml. (Hawk, Oser and Summerson, 1947). The low levels found in starfish are even more surprising in view of the rather high carbohydrate content of the clam portions fed, measured as containing at least 20 mg. of free reducing sugar. That this material is absorbed and retained by the starfish is shown in studies by Anderson (1952, 1953) and Greenfield *et al.* (1958), reporting that in addition to the large lipid depots, there are small but conspicuous glycogen stores in the digestive glands. Anderson further observed that these glycogen stores became depleted with fasting, indicating that they represent one of the animal's important nutrient constituents.

Earlier studies in the literature (*i.e.,* Lang and MacLeod, 1920; Van der Heyde, 1922, 1923b) report no reducing sugar present in the normal fluids of the starfish. New and better methods of analysis, however, enabled Greenfield *et al.* (1958) to observe concentrations of 10 to 50 μg. per ml. in the coelomic fluid of *Pisaster ochraceus.* This latter group also worked with *P. giganteus* but stated (p. 518), "The reducing sugar of the body fluid was present in such small quantities that it did not seem worth while determining." Van der Heyde concluded that echinoderms were in a constant state of sugar starvation, a view which has been echoed by more recent workers. The present study shows that in spite of the very low levels of reducing sugar found in the fluid, these levels are maintained fairly constant regardless of the nutritional state of the animal. Thus, the word "starvation" does not seem to suitably describe the condition.

The fact that the concentrations of reducing sugar are somewhat more constant than the nitrogen content in the coelomic fluid of starfish is in contrast to observations in other echinoderms. Lasker and Giese (1954) and Bennett and Giese (1955), working with the sea urchins, *Strongylocentrotus purpuratus* and *S. franciscanus,* discovered that reducing sugar levels show considerably greater variation between individuals than non-protein nitrogen values. These workers attributed the differences to the basic regulatory abilities of the animals towards the two substances. They supposed that the nitrogen content is fairly evenly regulated, while the sugar levels depend to a greater degree on the time since feeding. Somewhat similar observations were made by Tanaka (1958) on the sea cucumber, *Stichopus japonicus.* The data obtained in the present study indicate that no such differences in regulatory mechanisms occur in *A. forbesi,* which apparently must handle nutrients very differently from these other groups.

Translocation of ingested C^{14}-labeled nutrients

The levels of radioactivity found in the coelomic fluid of animals fed labeled nutrients are in full conformity with the observations on the nitrogen and sugar content of the fluid. No great peak in activity was seen after feeding, as occurs in the sea urchin (Farmanfarmaian and Phillips, 1962). Rather, low and fairly stable concentrations are present in the coelomic fluid at all times, with only slightly more elevated values soon after the initiation of the experiments. While the coelomocytes tend to increase gradually and somewhat erratically in activity, they do so only in the same proportion as the other tissues, and apparently show no indication of being special vehicles of nutrient transport. Some of the sharp fluctuations seen in the coelomocytes may be accounted for by errors in sampling, as no special care was taken to provide a constant "haematocrit" in each sample of fluid taken.

Distinct differences were noted between the effects of the nutrients administered. The amino acids of the labeled algal protein hydrolysate tended to be taken up by the coelomocytes to a much greater degree than either labeled glucose or palmitic acid. These cells are probably incorporating the amino acids into their own proteins. It is not clear whether they obtain the amino acids from the coelomic fluid or from contact with the digestive glands. In either case, it was almost a day before the activity of the coelomocytes surpassed that contained in the components dissolved in the coelomic fluid.

The partition of glucose-C^{14} activity between coelomocytes and fluid remained rather constant after the first day or so. It appears that glucose-C^{14} is taken up by the coelomocytes, but little of it is stored by these cells. Most likely, the absorbed glucose-C^{14} is rapidly metabolized and released back into the fluid as $C^{14}O_2$.

A distinctly different picture is seen in the animals fed palmitic-C^{14} acid. The activity levels measured in the fluid and coelomocytes were always very low. Furthermore, the greatest concentration of activity in the fluid occurred somewhat later than with the other nutrients, and the coelomocytes showed little tendency to increase in activity. The later fluid peak may represent a delayed penetration of the palmitic-C^{14} acid into the cells of the digestive gland. This substance is not normally one of the most easily absorbed foodstuffs. Once the palmitic-C^{14} acid had been taken up by the cells, it did not seem to be released again. The small amounts of activity that were observed in the fluid most likely represented metabolic products.

It has been suggested by several authors that if the coelomocytes do not function in the general transport of nutrient substances, they might at least be important in moving about special classes of compounds, such as lipids. This idea stems from the early investigators who observed these cells phagocytizing fat droplets and other substances. It now seems probable that most such phagocytosis represents a form of excretion. In the present study, small peaks of coelomocyte activity did occur soon after the feeding of palmitic-C^{14} acid. These might represent the efforts of some of the cells to phagocytize small droplets of nutrients encountered in their wanderings through the tissues. As the peaks quickly disappear, it is probable that these cells are disposed of in the usual manner—through the papulae. The fact that the coelomocyte activity remained consistently low after this initial period is evidence that these cells are not functioning in transporting lipids through the coelom. Whether or not an alternative route is being used cannot at this time be said.

Table I, showing the final distribution of activity in tracer-fed starfish, confirms previous reports that the digestive glands are the main storage organs present in the animals. Even at a week after the time of feeding, practically all the ingested material is still found in these structures. In Table II the distribution of activities in the various tissues has been put into terms of the organic (nitrogen) content of each of the tissues. The digestive glands, the coelomic fluid, and the coelomocytes all have comparable values. Possibly these elements have reached a kind of equilibrium with one another, while the other tissues sampled, the oral and aboral body walls, have not yet achieved this state. If this is the case, nutrients must have moved out of the digestive glands into the coelomic fluid and have been taken up from this medium by the other tissues.

An exception to this conclusion is seen in the animals fed palmitic-C^{14} acid. In spite of the large concentration of tracer found in the digestive glands, very little of it is seen anywhere else in the body. From this evidence, together with the previous observations, it is doubtful that lipids are readily mobilized from the digestive glands at all. It seems more likely that they are added to the general storage pools maintained in these organs, and are moved only after further transformation to some more easily distributed form.

A word of warning must be said in regard to this last conclusion, which is admittedly in need of further corroboration. The animals used in this study all possessed spent gonads. Farmanfarmaian *et al.* (1958) have demonstrated in *Pisaster ochraceus* that marked seasonal differences occur in the size of the digestive glands and gonads, the development of the two apparently being interrelated. Thus, there are times of the year when the gonads are growing at the expense of the material contained in the digestive glands. Furthermore, the fully developed gonads may possess a relatively large amount of lipid—up to 30% of their weight in *P. ochraceus* (Greenfield *et al.*, 1958). It is not yet known whether this lipid represents synthesis *de novo* in the gonad from non-lipid precursors, or material transported directly from the storage depots of the digestive glands. Thus, the animals in the present study might have been merely retaining lipids in their digestive glands for future translocation to the gonads when these later begin their development.

The experiments based on feeding starfish C^{14}-labeled nutrients have, then, thrown additional weight towards the argument that the coelomic cavity functions in nutrient transport. The various tissues of the starfish body can apparently partially or wholly satisfy their metabolic requirements by withdrawing nutrients from the coelomic fluid. The coelomocytes would seem to play no role in the process, or if they do have a role, they do not transport nutrients through the coelomic space. It is not yet clear whether additional mechanisms are also involved.

Translocation of injected C^{14}-labeled nutrients

The injection of nutrient substances into the coelomic cavities of starfish, in order to observe their absorption, is not a new idea. Van der Heyde (1922) performed several such experiments. He injected large quantities (up to 700 mg.) of either glucose or glycine into specimens of *Asterias* and then proceeded to withdraw 1-ml. samples of fluid from them at periodic intervals to measure the concentrations of these substances remaining. The nutrients disappeared rapidly from the fluid in a generally exponential manner, with the rate of removal decreasing with time. Rather similar results were obtained when the nutrients were added to a suspension of coelomocytes. These experiments indicate that the tissues, and particularly the coelomocytes, have a great affinity for amino acids and sugar. As the concentrations of the nutrients used by Van der Heyde were far in excess of those observed in the animals, it is not at all certain that this property is really significant in the normal transport of nutrients. Furthermore, Van der Heyde failed to recognize the exponential character of his curves. He attributed the various absorption rates obtained with different concentrations of injected substances to physiologically different conditions occurring in the tissue of the several animals used. In reality, the shapes of the curves indicate that materials are absorbed at rates essentially proportional to their concentrations in the coelomic fluid at any given time.

The present experiments were organized along somewhat different lines. C^{14}-labeled nutrients were employed so that the quantities of the injected substances in the fluid and various tissues could be followed, and only "physiological" doses needed to be used. As the tracers were injected in one ray and samples drawn from another, the time-course of their disappearance could not be followed. However, this procedure did permit the observance of a more normal appearance of the nutrients in the fluid of the sampled ray, and allowed an understanding of how the labeled substances were being handled by the various tissues.

It became clear that the nutrients were being removed from the coelomic fluid and appearing in the coelomocytes and other cells. Glycine-C^{14} and the amino acids in the algal protein-C^{14} hydrolysate showed a marked affinity for the coelomocytes and were taken up by them to high concentrations. This was exactly the same behavior observed with the fed animals, and further confirms that these cells obtain their nutrients directly from solution, and not by contact with the digestive glands. It is rather surprising that these same coelomocytes showed only a very low affinity for glucose-C^{14}, in spite of Van der Heyde's experiments in which they took up large quantities of glucose. It would seem from the present data that coelomocytes normally do not store great quantities of nutrients, but take in only what they require to satisfy their own metabolic needs.

The results presented in Table III indicate that all the tissues of the starfish are able to absorb nutrients from the coelomic fluid in spite of the very low concentrations found there. While most of the activity was taken up by the digestive glands, these organs represent the largest mass of organic tissue in the animals, and they have a very large surface area for exchange. Actually, as seen in Table IV, it was the coelomocytes that showed the greatest affinity for the tracers. As they are suspended in the fluid and have the greatest surface-volume ratio, this result should be expected.

The data presented in Table IV are also of interest in another respect. They suggest, at least for the specimens injected with glucose-C^{14}, that all the coelomic fluid of the animals has come into equilibrium with the activity taken up by the digestive glands in the injected rays. The fluid in the uninjected rays has a considerably higher activity per unit of nitrogen than the digestive glands or other tissues found there. It seems that there must be material being transferred *via* the coelomic fluid from the "hotter" organs to those containing less activity. There is a tendency, then, for the nutrients to achieve an even distribution throughout the animals and for all the tissues to approach a type of equilibrium in respect to these substances. For this to be true, there must be a fairly rapid flux of molecules into and out of the tissues, with each tissue continually picking up new material in exchange for old, while still maintaining itself in a relatively unchanged state.

The results of these experiments thus complement those found in the literature. Not only do they further implicate the coelomic fluid as the most important medium for nutrient transport, but they also suggest that transport is a two-way process. Nutrients are not just carried to distant tissues for utilization, but are continually being exchanged back and forth among the different tissues through the coelomic fluid. Each tissue may be said to be in a condition of dynamic equilibrium (or steady-state) with the others. More concrete evidence for this dynamic state and its role in nutrient transport will be presented in a subsequent paper (now in preparation).

SUMMARY

1. A series of investigations was undertaken in an effort to clarify the role of the coelom and the coelomic fluid in the transport of nutrient substances throughout the body of starfish.

2. Serial chemical analyses of organic substances in the cell-free coelomic fluid of individuals did not reveal significant differences between fed specimens and those that had been starved for at least one week. The mean value of total nitrogen was 63.5 μg. N per ml., but large fluctuations occurred in all the specimens. Free ammonia was found to be present fairly constantly in the fluids, with a mean value of 12.8 μg. N per ml., or about 20% of the total nitrogen. Protein was determined to represent about 25% of the total nitrogen in two analyses. Reducing sugar occurred in the coelomic fluids in extremely low concentrations (mean value = 6.9 μg. per ml.) and showed moderate fluctuations in individual specimens.

3. Experiments in which starfish were fed C^{14}-labeled algal protein hydrolysate, glucose, and palmitic acid showed that these substances were stored in the digestive glands and reached the other tissues only very slowly. Except possibly for palmitic acid, low levels of these nutrients occurred consistently in the coelomic fluid and coelomocytes at all times following feeding.

4. Experiments in which small amounts of C^{14}-labeled algal protein hydrolysate, glycine, and glucose were injected into the perivisceral coelom of starfish revealed that these substances are readily absorbed by the different tissues of the body, especially the digestive glands and, to a much lesser extent, the coelomocytes. An even greater proportion of glucose than the amino acids was taken up by the digestive glands.

5. These studies implicate the coelomic fluid as the most important medium of transport, and suggest that transport is accomplished as part of a continual flux of substances between the coelomic fluid and the various tissues of the body.

LITERATURE CITED

ANDERSON, J. M., 1952. Cellular structure and function in the digestive diverticula of the starfish, *Asterias forbesi*. *Anat. Rec.*, **113**: 541.

ANDERSON, J. M., 1953. Structure and function in the pyloric caeca of *Asterias forbesi*. *Biol. Bull.*, **105**: 47–61.

BENNETT, J., AND A. C. GIESE, 1955. The annual reproductive and nutritional cycles in two western sea urchins. *Biol. Bull.*, **109**: 226–237.

BOOLOOTIAN, R. A., 1962. The perivisceral elements of echinoderm body fluids. *Amer. Zool.*, **2**: 275–284.

Boolootian, R. A., and A. C. Giese, 1958. Coelomic corpuscles of echinoderms. *Biol. Bull.*, **115**: 53–63.

Budington, R. A., 1942. The ciliary transport-system of *Asterias forbesi*. *Biol. Bull.*, **83**: 438–450.

Chapeaux, M., 1893. Sur la nutrition des échinodermes. *Bull. Acad. Roy. Belgique*, **26**: 227–232.

Cohnheim, O., 1901. Versuche über Resorption, Verdauung und Stoffwechseln von Echinodermen. *Zeitschr. f. Physiol. Chem.*, 33: 9–54.

Cuénot, L., 1887a. Études sur le sang, sa rôle et sa formation dans le série animale. *Arch. de Zool. Exp.*, **2**: XLIII–XLVII.

Cuénot, L., 1887b. Contribution à l'étude anatomique des astérides. *Arch. Zool. Exp. et Gen., sér.* **2**, **5**: 1–144.

Delaunay, H., 1931. L'excrétion azotée des invertébrés. *Biol. Rev.*, **6**: 265–301.

Farmanfarmaian, A., and J. H. Phillips, 1962. Digestion, storage, and translocation of nutrients in the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **123**: 105–120.

Farmanfarmaian, A., A. C. Giese, R. A. Boolootian and J. Bennett, 1958. Annual reproductive cycles in four species of west coast starfishes. *J. Exp. Zool.*, **138**: 355–367.

Feder, H. M., 1956. Natural history studies on the starfish *Pisaster ochraceus* (Brandt, 1893) in the Monterey Bay area. Doctoral Dissertation, Stanford University.

Giordano, M. F., H. A. Harper and F. P. Filice, 1950. The amino acids of a starfish and a sea urchin (Asteroidea and Echinoidea). *Wasmann J. Biol.*, **8**: 129–132.

Greenfield, L., A. C. Giese, A. Farmanfarmaian and R. A. Boolootian, 1958. Cyclic biochemical changes in several echinoderms. *J. Exp. Zool.*, **139**: 507–524.

Hawk, P., B. Oser and W. Summerson, 1947. Practical Physiological Chemistry. Blakiston Co., Philadelphia.

Irving, L., 1924. Ciliary currents in starfish. *J. Exp. Zool.*, **41**: 115–124.

Lang, R. S., and J. J. R. MacLeod, 1920. Observations on the reducing substances in the circulatory fluids of certain invertebrates and fishes. *Quart. J. Exp. Physiol.*, **12**: 331–337.

Lasker, R., and A. C. Giese, 1954. Nutrition in the sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.*, **106**: 328–340.

Myers, R. G., 1920. A chemical study of the blood of several invertebrate animals. *J. Biol. Chem.*, **41**: 119–135.

Park, J. T., and M. J. Johnson, 1949. A submicrodetermination of glucose. *J. Biol. Chem.*, **181**: 149–151.

Somogyi, M., 1942. Determination of blood sugar. *J. Biol. Chem.*, **160**: 69–73.

Tanaka, Y., 1958. Feeding and digestive processes of *Stichopus japonicus*. *Bull. Fac. Fish. Hokkaido Univ.*, **9**: 14–28.

Van der Heyde, H. C., 1922. On the Physiology of Digestion, Respiration and Excretion in Echinoderms. Academical Dissertation, Amsterdam.

Van der Heyde, H. C., 1923a. Petites contributions à la physiologie comparée II La resorption chez les échinodermes. *Arch. Néerland. de Physiol.*, **8**: 151–160.

Van der Heyde, H. C., 1923b. Petites contributions à la physiologie comparée IV Sur l'excretion chez les échinodermes. *Arch. Néerland. de Physiol.*, **8**: 151–160.

White, A., P. Handler, E. L. Smith and D. Stetten, 1954. Principles of Biochemistry. McGraw-Hill Book Co., Inc., New York.

WATER BALANCE IN ANOMURAN LAND CRABS ON A DRY ATOLL

WARREN J. GROSS

Division of Life Sciences, University of California, Riverside, California

Degrees of the terrestrial habit have been achieved by many representatives of the decapod Crustacea. Still, none are completely successful, for they are all bound to water by their reproductive habits. Some of the most successful invaders of land in this group are anomuran crabs, which despite their abundance and common occurrence in tropical regions have received little attention with respect to the physiological adaptations, especially those concerned with water regulation, which make life on land possible for them. Edney (1960) reviews the subject of terrestrial adaptations among the Crustacea. Gross (1955) demonstrated in laboratory experiments that the anomuran cocoanut crab, *Birgus latro,* can control its blood concentration by selecting water of appropriate salinity, and when given a choice it will visit fresh water more often than sea water. Gross and Holland (1960), on the other hand, demonstrated a similar behavioral mechanism in the hermit crab, *Coenobita perlatus,* but this species showed a definite preference for sea water over fresh water when offered a choice.

The above mentioned investigators also demonstrated tolerance to a wide range of blood osmotic concentrations in *Birgus* and *Coenobita.* Yet no information is available concerning the range of variation experienced by these crabs in their natural environments. On the other hand, Harms (1932) made a few freezing point determinations on the blood of *Birgus* and Pearse (1934) did the same on *Coenobita clypeatus,* but these workers were not explicit as to the environmental conditions under which the blood samples were taken from the animals.

The field studies of present investigation were conducted at the Eniwetok Marine Biological Laboratory at Eniwetok Atoll, Marshall Islands, during the months of February and March of 1963. This is typically the dry season of the year, each of these two months having a normal precipitation of less than 1.9 inches whereas the normal annual rainfall is about 50 inches (Arnow, 1954). This time of the year, then, would usually be a critical period for land crabs which depend on water sources other than the sea, but the particular period during which this study was conducted was even more critical than usual because the February precipitation was only 0.19 inch and throughout March, up to the 27th when field studies were terminated, the precipitation was only 0.28 inch.

This study reveals the range of blood concentrations endured by three species of terrestrial anomuran crabs during a drought and furnishes information concerning their sources of water under the stressed conditions of the dry season. Eniwetok Atoll is especially suitable for this study because its islets present different extremes in environment from well wooded conditions, rich in detritus, to exposed conditions of poor vegetation and dry soil consisting mostly of coral sand.

MATERIALS AND METHODS

Three anomuran crabs of the family Coenobitidae were studied in the field and in the laboratory: The cocoanut crab *Birgus latro* Linnaeus, and the hermit crabs, *Coenobita perlatus* Milne-Edwards and *Coenobita brevimanus* (Dana).

All three species were studied in the laboratory at Eniwetok under conditions which are described below. However, a group of *Coenobita brevimanus* was flown to Riverside where it was maintained for several weeks in cages in a greenhouse where the temperatures varied between 20° and 33° C. Here the animals had a choice of sea water or fresh water made available in open dishes or dispensers which permitted estimation of the volume of water used. Also available was a box filled with a mixture of peat moss and sand, which was maintained in a damp condition with fresh water. This was to simulate the damp detritus of cocoanut piles where this species was captured. Cocoanut and fish meal were made available for food. Thus, the laboratory conditions under which *C. brevimanus* was maintained in Riverside are believed to approximate those it encounters in nature with respect to water relations, and hereafter shall be referred to as "normal." All animals used in this investigation were mature and between molts. The smallest specimens of hermit crab weighed approximately 20 g. without the shell. The carapace width of *Birgus* ranged between 4.5 cm. and 15.0 cm. Sexes were equally divided for *Birgus*. However, note could not be made of sex for most *Coenobita* in the field because of the difficulty in removing the crabs from their shells. On the basis of a token sampling, the sexes seemed about equally divided for both species of *Coenobita*.

Blood was extracted in the field from all three species by puncturing the arthrodial membranes at the joints of the appendages by means of glass pipettes. The blood was introduced into vials which in turn were placed into a Thermos bottle filled with ice held in a plastic bag to prevent water from leaking into the screw-cap vials. Such samples remained cold until returned to the laboratory at Eniwetok for analysis.

Urine was extracted only in the laboratory because this required immersion in cold water to relax the animal, a treatment from which it readily recovered. Care was taken to dry the nephropores before inserting the cannula.

Osmotic concentrations of body fluids were determined by means of a Mechrolab vapor pressure osmometer. In the case of blood this required the removal of the clot to prevent coagulum from adhering to the thermistor of the osmometer. One reading by means of the above osmometer on uncoagulated whole blood of *C. brevimanus* gave the same value as the serum of the same sample within the precision of measurement of the instrument, which has less than a 2% error with the methods used in this investigation. Also, Prosser *et al.* (1955) and Gross (1963a) have demonstrated no osmotic difference between blood and serum of *Pachygrapsus* and *Hemigrapsus*, respectively. There is good reason, therefore, to assume no difference between the osmotic concentration of blood and serum for all species examined in this study.

Salinities of water (both surface water and crab shell water) were estimated in the field by means of an American Optical Company TS meter (Goldberg refractometer). This instrument can be carried conveniently in the pocket and is self-correcting for temperatures between 15° and 37° C. The error was less than

3% in the range of normal sea water (3.43% salt). This standard for normal sea water is a common salinity in the coastal waters near Riverside. However, sea water from the open lagoon at Eniwetok was observed to be 104% of the above standard.

RESULTS

Both *Birgus* and *C. brevimanus* were found only in secluded, protected situations. In all cases *C. brevimanus* was found in areas of heavy vegetation, burrowed in piles of rotten cocoanuts often associated with *Birgus.* This hermit crab was never found in or near the sea. *Birgus,* with the exception of those specimens found on Jieroru Islet (to be discussed below), also was observed to be confined to densely vegetated areas near or in piles of rotten cocoanuts. The piles of cocoanuts are rich in detritus. In their depths they were damp to the touch despite the recent paucity of rain. *Coenobita perlatus* is by far the most common of the three subjects at Eniwetok. It is commonly found in exposed situations close to the edge of the sea, where it aggregates in large numbers during the day among the roots of trees and shrubs (*Messerschmiddia* and *Scaevola*). Young specimens of *C. perlatus* were found on islets completely devoid of vegetation, usually aggregated in the daytime under wreckage or debris. However, this species was commonly observed in the forested areas, particularly at night when it was most active. It occasionally was found burrowed in piles of rotten cocoanuts along with *Birgus* and *C. brevimanus,* but this was not common. *C. perlatus* was observed to enter brackish water of pools and sea water of the lagoon. It is of interest that the largest aggregation of this species found was at the edge of a pool of brackish water (58% sea water) which was in a compartment of a wrecked barge. During this study the only standing fresh water observed was shortly after a rain squall, in the form of droplets on leaves. This was short-lived and was observed only once, although during the two-month drought period local rain squalls of short duration were common throughout the atoll.

Figure 1 illustrates the serum osmotic concentrations of the three species collected from different environmental situations. The blood samples for *Birgus* and *C. brevimanus* from the cocoanut piles were taken on Igurin and Giriinien Islets during the day, and samples from the two sites for both species were not significantly different from each other. However, the mean concentration for *Birgus,* 74.7% sea water (Fig. 1, A), was significantly less than the mean value for *C. brevimanus,* 80.3% sea water (Fig. 1, C), $P < 0.01$. The value for *Birgus* compares closely with that given by Harms (1932) although the conditions in which the animals were found or kept are not described explicitly by this worker. On the other hand, the sera of three specimens of *Birgus* collected in the daytime from deep dry burrows on Jieroru Islet were 88.4, 91.2 and 95.2% sea water (Fig. 1, B). This islet is relatively bare of vegetation; all palm trees have been destroyed and detritus is scarce. The crab burrows, which were deep and well sealed in two cases, were dug in a substrate which was dusty and dry to the touch. The only apparent source of water was the lagoon. All three crabs were active and seemed to be in excellent condition.

As mentioned above, *C. brevimanus* was found only in cocoanut piles where the substrate was damp. It was not observed foraging at night, as were the other two species, but such activity was probably overlooked because of the relatively small population of *C. brevimanus*. Blood samples were taken from the cocoanut crabs only in the daytime when they probably had been removed from any source of surface water for hours. The values for blood concentration in this species (Fig. 1) are thus believed to be maximum.

Coenobita perlatus, however, was found in a variety of environmental situations which is reflected in the wide range of serum osmotic concentrations. Figure 1 reveals the mean serum concentrations for the following environments: exposed and inactive close to lagoon's edge in daytime, 124% sea water (Fig. 1, D); filling shells with lagoon water at night, 128% sea water (Fig. 1, E); active in wooded area at night, 128% sea water (Fig. 1, F); filling shells with brackish water at night, 115% sea water (Fig. 1, G); inactive and burrowed in piles of rotten cocoanuts in daytime, 106% sea water (Fig. 1, H). It should be pointed out that in both

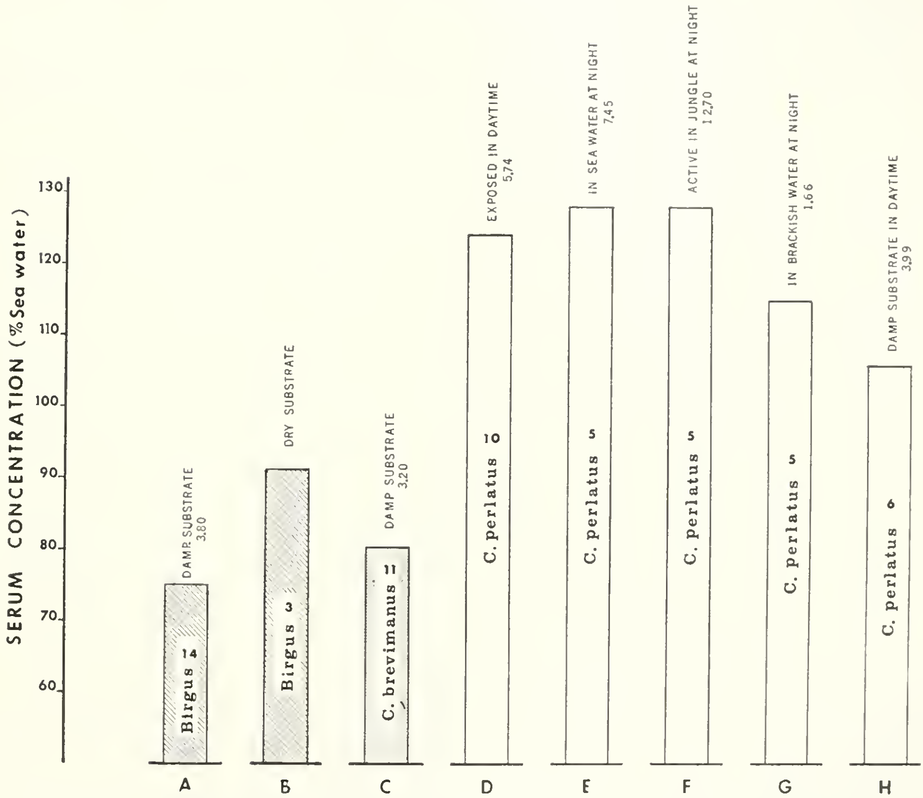


FIGURE 1. Osmotic concentration of serum sampled in different field conditions from terrestrial crabs. Height of bar represents mean serum concentration. Numeral inside bar indicates number of cases. Numeral above bar represents standard deviation of the mean. All three values for the Jieroru *Birgus* (B) are given in text. Brief descriptions of environments are indicated over bars. Details of conditions are given in the text.

groups which were filling their shells (E and G, Fig. 1), about one-half hour was allowed to lapse after capture before blood samples were taken. This was to permit any possible equilibrium to take place between shell water and blood following the filling process. The lowest blood concentration observed for this species was 102% sea water from a crab taken in a pile of cocoanuts; the highest observed was 150% sea water from an active crab which was foraging in the wooded area at night. Thus, *C. perlatus* maintains its blood concentration considerably higher than *Birgus* or *C. brevimanus*, even when found in similar environments where the availability of water for all three species is the same (*e.g.*, wooded areas and burrowed in cocoanut piles). On the other hand, *C. perlatus* does not maintain such uniform serum concentrations as the other two species when it has equal opportunities with respect to the availability of water.

When *C. perlatus* was exposed exclusively to sea water for more than two weeks in the laboratory, the concentration of its blood, as calculated from total cations, averaged less than 110% sea water (Gross and Holland, 1960). The higher values observed in the field, therefore, suggest that significant evaporation takes place during prolonged absence from a source of water in the field.

TABLE I

Shell water concentrations in Coenobita captured in the field

Species	Collecting site and conditions	Concentration of nearest observed surface water (% sea water)	No. cases	Mean shell water concentration* (% sea water)
C. perlatus	Japtan Islet; edge of lagoon under board	104	6	125 (119–140)
	Igurin Islet near sump under board	76	3	105 (104–106)
	Igurin Islet in compartment of wrecked barge high on beach of lagoon	58	5	97 (87–111)
	Igurin Islet; active in wooded area	104	5	125 (117–134)
	Igurin Islet in water at edge of lagoon	104	5	107 (104–108)
	Jieroru Islet; inactive under roots of trees at edge of lagoon	104	7	126 (109–131)
	Giriinien Islet from piles of cocoanuts	104	3	115 (105–134)
C. brevimanus	Giriinien Islet from piles of cocoanuts	104	5	80 (73– 87)

* Values in parentheses indicate observed range.

In all observed field cases both *Birgus* and *C. brevimanus* maintain their serum concentrations below the concentration of the nearby sea; even *Birgus* from Jieroru was thus.

No obvious trends in the serum concentration were observed with respect to sex or size in any of the species examined.

The concentration of water carried in the shells of the hermit crabs was measured in the field in order to reveal something about the sources of water for these crabs in nature. Table I presents the observed concentration for shell water taken from the two species of *Coenobita* from different environmental conditions. It should be pointed out that in many cases water could not be taken from the crab's adopted shell. For example, only 5 out of 21 specimens of *C. perlatus* from the wooded area yielded water. This might be expected because of the relatively great distance to the sea. The salinity of the shell water, unless recently filled, was higher than the sea and was close to the concentration of the animal's blood. Thus, the mean ratio, shell water concentration/serum concentration, for five cases of *C. perlatus* from which both samples were taken was 1.04; range: 1.02–1.09. Obviously this ratio would be different in a crab which was in the process of filling its shell. As mentioned above, this species was observed filling its shell at night, both with sea water at the edge of the lagoon and with brackish water from a pool. It is interesting that shell water from all crabs captured in the morning, close to the lagoon's edge (Jieroru Islet), was highly concentrated. This suggests that an osmotic equilibrium was established between shell water and blood because there had been little opportunity for evaporation of the shell water which probably had come from the sea only a few hours earlier.

TABLE II

Shell water and serum concentration of Coenobita brevimanus maintained in the laboratory under different conditions

Condition	Period of exposure (days)	Mean shell water concentration (% sea water)*	No. cases	Mean serum concentration (% sea water)	No. cases	S. D.
I						
F.W., S.W. and damp peat moss "normal"	21	76.4 (67.0–84.6)	5	79.7	5	1.45
	>28	74.2 (64.2–104)	7	—	—	—
II						
S.W. only and damp peat moss	2	95.3 (87.5–99.2)	3	—	—	—
	5	85.8 (64.2–104)	4	95.6	5	7.16
	>15	115 (102–131)	5	—	—	—
III						
Damp peat moss only	>15	no water		86.4	6	10.2

* Values in parentheses indicate observed range.
F.W. = fresh water, S.W. = sea water.

The shell water of *C. brevimanus*, on the other hand, was dilute to the sea in all five field cases observed (Table I). Still, no source of surface brackish water or fresh water could be found within several hundred meters of the collecting area. Again, however, the concentration of shell water was close to isotonicity with the blood of *C. brevimanus*. Large quantities of shell water are commonly found in *C. brevimanus*. Unfortunately, when the largest aggregation of this relatively uncommon species was found (on Japtan Islet), there was no available means of measuring the salinity of the shell water. Again, however, the only visible source of surface water was the sea which was at least 200 meters away.

Now there is no doubt that *C. perlatus* fills its shell with available surface water (Table I); however, no such process was observed for *C. brevimanus*. With the relative scarcity of this species on the islets studied, however, such a process easily could have been overlooked. Table II presents the results of experiments performed in the laboratory at Riverside, to determine the source of shell water in *C. brevi-*

manus. Thus, in Group I, which was given access to surface sea water, fresh water and peat moss dampened with fresh water, the shell water averaged about 75% sea water; after three weeks in such a situation the osmotic concentration of the blood averaged 79.7% sea water, as compared to the average 80.3% sea water for *C. brevimanus* sampled in the field. It thus seems that the laboratory condition termed "normal" in this investigation does indeed approximate field conditions.

Group II, on the other hand, which was presented only free sea water and damp peat moss, showed low concentrations for shell water for at least 5 days after transfer from "normal" conditions. However, after 15 days' exposure to this condition, the salinity of the shell water in every observed case was higher than the available normal sea water. The low concentration of shell water during the early part of this experiment, then, must have been a residue from the previous "normal" condition where fresh water was present, and/or a matter of dilution of the normal sea water taken into the shell by the water from a hypotonic blood which diffused outward through the body wall. Also, it will be noticed (Table II) that the blood osmotic concentration for this group averaged close to that of the available free sea water (95.6% sea water). The lowest value in this experimental group (87.6% sea water) was higher than the highest value observed for any field sample (85.2% sea water). All animals were in apparently good condition at the end of this experiment, thus indicating tolerance to elevated blood concentration. There is evidence, then, that *C. brevimanus* will use sea water to fill its shell when fresh water is not available. It follows, however, that the low osmotic concentration of shell water and serum observed in the field samples is not the result of diluting sea water with fresh water which is absorbed from the substrate.

Animals of Group III remained viable for more than two weeks in the absence of any surface water. These persistently burrowed in the damp detritus which they introduced into their shells; they fed, and were active at the termination of the experiment. However, shell water could not be obtained from this group. Thus, it does not seem likely that the large quantities of shell water observed in this species in the field could have been absorbed from the damp substrate and then secreted into the adopted shell. While the serum concentration of this group averaged 86.4% sea water, which was somewhat higher than the field samples as well as the laboratory crabs from the "normal" condition (Group I, Table II), the range was 74.8–104% sea water. This is a higher maximum and a lower minimum than observed for the field samples (74.9 and 85.2, respectively).

It was observed following this treatment that these crabs completely drained two watering devices in less than 15 hours, one containing about 500 ml. of fresh water, the other about 200 ml. of sea water. Such quantities obviously could not be consumed internally by six crabs. Rather it was taken into the shell repeatedly and spilled outside of the watering troughs. Six animals maintained in the "normal" condition consumed less than 100 ml. of fresh water daily and only traces of sea water. Such a behavior suggests that the crabs had become dehydrated in the absence of free surface water, but since the blood concentrations remained relatively low, it also suggests that salts were lost by the crab to the damp substrate. It is apparent, nevertheless, that *C. brevimanus* could survive, burrowed in rotten cocoanut piles without access to fresh water during an extended drought such as is common at Eniwetok.

Similarly, when crabs of Group II were given a choice of fresh water or sea water after having been deprived of fresh water for more than two weeks, they drained the fresh-water dispenser of about 500 ml. in a period of four hours. No loss of sea water could be detected from its respective dispenser. After a period of 15 hours 800 ml. of fresh water had been used, even though the position had been exchanged with sea water, and although there were indications that the crabs had visited sea water, no detectable amount had been used. These experiments strongly suggest a decided preference for fresh water by this species and further suggest a behavioral mechanism for maintaining proper water balance such as that described for *Birgus* by Gross (1955) and for *Coenobita perlatus* by Gross and Holland (1960).

Hydration and dehydration in hermit crabs could not be measured by weight changes because forceful removal of the animal from its adopted shell caused loss of fluid which could not be measured with precision. Measurement of weight changes of the animal in its gastropod shell were of no value because, as mentioned above, undetermined amounts of solid material were habitually introduced into the shell by the animal.

Figures 2 and 3 illustrate the results of experiments conducted in the laboratory at Eniwetok concerning the effects of available fresh water or sea water on the serum osmotic concentration of *C. brevimanus* and *Birgus* in a situation where they could not burrow but where they could readily reach water in an open dish, as well as a supply of fresh cocoanut. Thus, 9 days following transfer from a condition where both fresh water and sea water were available to where only fresh water was present, the serum concentration of *C. brevimanus* was slightly higher on the average than the mean of the field samples; after 17 days the serum concentration was slightly lower than the field samples (Fig. 2). But 5 days after fresh water was replaced with sea water, the blood concentration rose dramatically, all five cases being far higher than anything observed in the field.

A similar phenomenon was observed on two specimens of *Birgus*. It will be noted in Figure 3 that the serum of one specimen rose in concentration considerably above its field value, even after it was exposed exclusively to fresh water for more than two weeks. This possibly was caused by the crab's inability to burrow, thus permitting water loss from the body surface through evaporation. The second specimen also demonstrated a slight increase in its blood concentration and while it, too, was unable to burrow into a damp substrate, it was the larger specimen of the two which may have allowed it greater physiological stability in the above captive condition. Nevertheless, both specimens, when exposed exclusively to normal sea water for five days, demonstrated increases in the serum concentration to levels which were matched only by crabs sampled in the dry situation on Jieroru Islet. Therefore, when only sea water is available, *Birgus* is unable to maintain the low blood concentrations observed in the field on Igurin and Giriinien Islets. Also, there is evidence that the burrowing behavior of this species helps to maintain the low blood concentrations observed in the field. This could act in preventing water loss by evaporation and/or permitting absorption of water from the substrate in a way similar to that demonstrated by the brachyuran land crab, *Gecarcinus* (Bliss, 1956).

The osmotic concentration of urine on all three species was also determined.

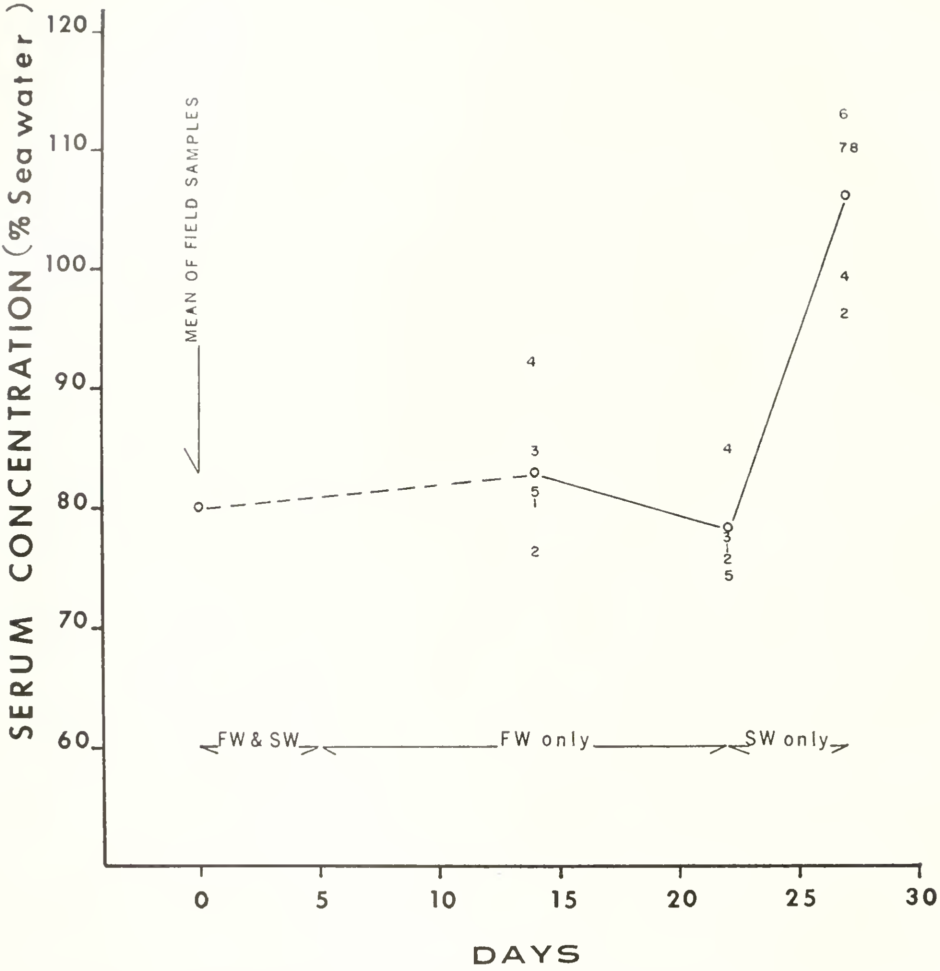


FIGURE 2. Response of *Coenobita brevimanus* to water of different salinity under laboratory conditions. Available water for different periods is indicated at the bottom of the graph. SW = normal sea water; FW = fresh water. Numerals represent individual specimens. Open circles represent mean values.

Thus, the ratio, urine concentration/blood concentration (u/s), for *C. perlatus* averaged unity for five specimens (range 0.98–1.02). One specimen had been exposed to both sea water and fresh water, for two days after capture. The other four specimens had been exposed to fresh water for two weeks. As seen by the range, the treatment made no difference. Four specimens of *C. brevimanus* exposed to fresh water for one to two weeks had a mean u/s value of 0.97 (range: 0.94–0.99). Attempts failed to extract urine from both species of *Coenobita* after they were exposed exclusively to sea water.

Only two u/s values were determined on individual specimens of *Birgus*. When

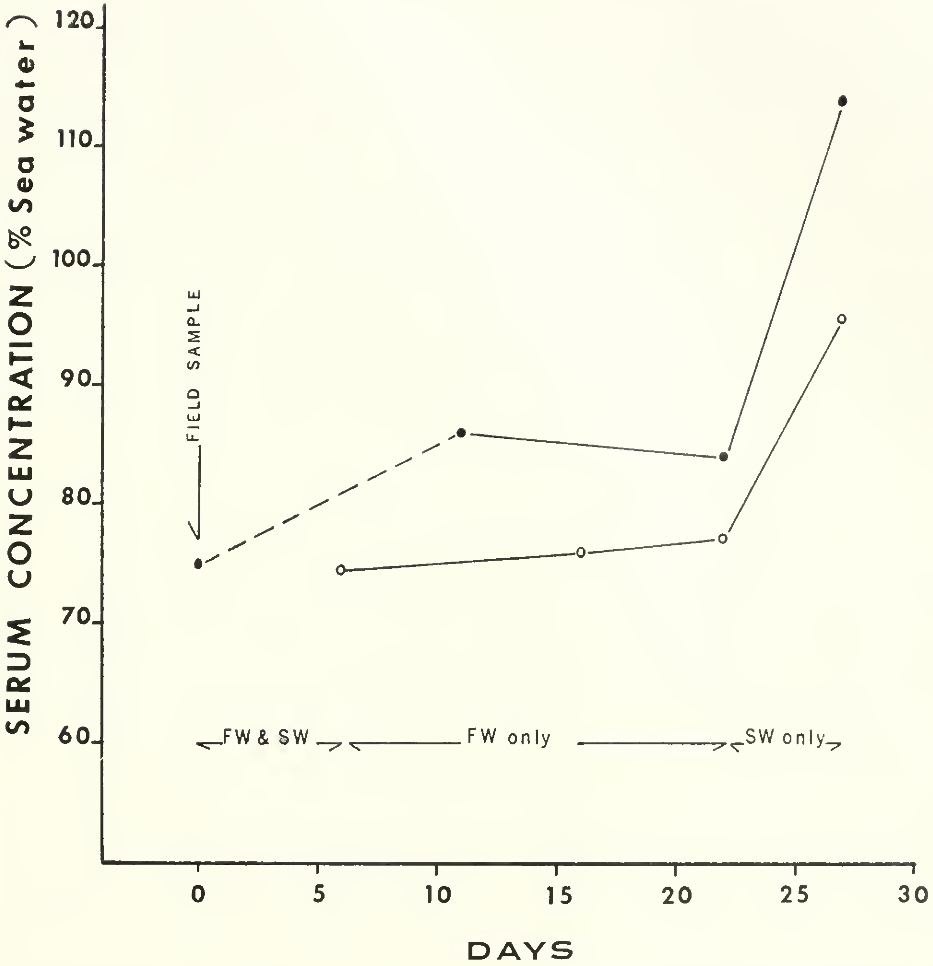


FIGURE 3. Response of two specimens of *Birgus* to water of different salinity under laboratory conditions. Line joining solid circles represents history of smaller crab. Line joining open circles represents history of larger crab. Available water for different periods is indicated at the bottom of the graph. SW = normal sea water; FW = fresh water.

one crab was exposed to sea water and fresh water for six days after capture, its u/s value was 0.95. The other specimen, which was exposed to the same conditions for only one day after capture, had a value of 0.82. However, urine samples from 5 crabs exposed to both sea water and fresh water for one day after capture averaged 66.7% sea water, which is less than the mean serum concentration for *Birgus* sampled in the field (74.7% sea water). There is a tendency, therefore, for the urine of both *C. brevimanus* and *Birgus* to be hypotonic to the blood. It is doubtful, however, that such a low degree of hypotonicity has any physiological significance. There thus is no evidence that the antennary glands of any of the three species are specially adaptive for terrestrial life. However, the small amounts of urine which could be extracted may indicate a low urine output, which would mean conservation of water. Loss of salt by way of the urine likely would present little problem to such a land animal inhabiting an environment exposed to sea spray.

Observations were made in the field on temperature and humidity. Thus, daytime relative humidities ranged between 60% and 80%; air temperatures about four feet above ground (including day and night) ranged between 25.0° and 33.2° C. and substrate temperatures between 26.0° and 28.5° in areas where crabs were captured. Body temperatures of *Birgus* captured in the daytime ranged between 26.7° and 30.0° C. There seemed, therefore, to be no critical stress imposed by these two physical factors on the land crabs of this area. Evaporation doubtless could occur from the body surface, especially if the crabs were exposed to the fresh tradewinds which blow constantly at this time of the year. However, the hermit crabs are protected from such evaporation by their adopted shells and *Birgus* is usually found in covered areas where it is shielded from the wind.

DISCUSSION

Seurat (1904) reported that *Coenobita perlatus* in Mangareva habitually migrates to the sea at night to replenish its supply of water which it carries in its shell. Such a behavioral device might have initiated extended stays on land by the early terrestrial hermit crabs. The present investigation confirms that *C. perlatus* will fill its shell with sea water, but also that it will visit brackish water (58% sea water) for the same end. No standing fresh water could be found in the field during this investigation. However, Gross and Holland (1960) demonstrated that this species will enter fresh water in the laboratory, and this in turn effects a lower blood concentration than those observed in the present investigation. Thus, it is predicted that during the rainy season when there is an abundance of standing, fresh water, the average blood concentration of this species would be considerably lower. The large aggregation of *C. perlatus* found around a brackish pool as mentioned above cannot be interpreted as necessarily a preference for this salinity; rather it may reflect a preference for the calmness of the pool over the turbulence of the edge of the lagoon, where hermit crabs are tumbled violently by the lapping waves as they fill their shells.

It seems unlikely that any of the *C. brevimanus* sampled in the field depended on the sea for water. As shown in Figure 2 and Table II, exclusive access to sea water for 5 days elevates the blood concentration above anything observed in the field. Also, Table II shows that shell water of animals exposed to only sea water was much higher than that observed in the field animals.

Inasmuch as this species does not secrete large volumes of fluid into the shell, in the absence of surface water (Table II), the substantial quantities of shell water of low salinity observed in the field must have come from a source of fresh or brackish water which was not found within hundreds of meters of the site of capture. Now the shell water of 5 laboratory specimens of *C. brevimanus* averaged 60% sea water when only fresh water was available. Thus, the salinity of the shell water does not reveal the concentration of the water used by the crab, but it does indicate hypotonicity to sea water, however, and this excludes the sea as the sole source of water to the animal. Obviously, in the above case, the shell water concentration of 60% sea water indicates that body salts diffused into the shell. While the source of water for these animals in nature is uncertain, on one occasion a specimen of *C. brevimanus* was observed inside the shell of a rotten cocoanut which held fluid of an osmotic pressure equivalent to 35% sea water. Such a supply of water may be common in the wooded areas where both this species and *Birgus* could reach it as well as cocoanut milk.

Experiments in the laboratory demonstrated that *C. brevimanus* can fill its shell from a puddle which is only about 4 cm.^{2} in area and 1 cm. deep. Such a small source of water may have been common in the wooded areas but also would be easy for the investigator to have missed.

The evidence also suggests that *Birgus* does not depend on the sea for its source of water when it lives in the detritus of piles of rotten cocoanut. As shown in Figure 1, A, the blood concentration of such crabs was found to be greatly less than sea water, yet when *Birgus* is given access to only sea water, its blood elevates dramatically (Fig. 3). The source of water for *Birgus* on the dry, exposed islet of Jieroru seems more likely to be the sea water of the lagoon. The blood concentrations of all three specimens captured there were high. The soil was dry and, at the time the specimens were captured, unlikely to yield water to the body surface of the animals. There were no cocoanut trees and very little protection from the wind on the surface of the islet. The sparse vegetation was dry and exposed to sea spray, and thus unlikely to furnish adequate water if ingested. Still, these animals appeared normal and were very active. Harms (1932) reports that *Birgus* can obtain water from dew. This could be true in the wooded protected areas, such as found on Igurin and Giriinien Islets. It seems unlikely, however, that sufficient dew could condense on the windswept, exposed islet of Jieroru to supply *Birgus* with adequate water of low salinity. The serum concentrations of the animals sampled there attest to this. Dr. Edward Held (personal communication) has observed *Birgus* on the reef flat of Mui Islet at Eniwetok and captured two males whose tracks indicated they were returning from the reef flat to the wooded area. He has not observed *Birgus* at any time actually in sea water. It is reported that only females of adult *Birgus* re-enter the sea once they emerge, and then only to deposit their eggs (Harms, 1932). However, the cocoanut crab has been observed to drink sea water in captivity and was kept in an active condition exclusively on sea water for 78 days, after which time the blood osmotic concentration was equivalent to 118% sea water (Gross, 1955). This same investigation demonstrated that *Birgus* prefers fresh water to sea water, but will enter the latter occasionally if offered a choice between the two; the blood concentration of *Birgus* when given access to both fresh water and sea water was equivalent to about 90% sea water, which is considerably higher than the field samples taken in the wooded area of Igurin and Giriinien Islets during the present investigation. It is concluded that *Birgus* captured near cocoanut piles had sources of water of low salinity and did not depend on the sea for its water supply.

On the other hand, the only obvious reliable source of water for the Jieroru crabs during this drought period was the sea, and as shown above, this species can live for a sufficiently long period on sea water to survive the dry season. An alternative explanation for the high blood concentrations of the Jieroru crabs is that they were tolerating partial desiccation. It is possible that they could endure the dry season by such tolerance to desiccation but also by keeping water loss by evaporation at a minimum by sealing the entrances to their burrows; this was observed for two of the crabs captured on this dry islet.

The range of blood concentrations for *Birgus* and *C. brevimanus* captured on Giriinien and Igurin Islets is remarkably small compared to that of *C. perlatus*, which is not as discriminating in its choice of habitat as the other two species. The high degree of osmotic homeostasis observed in *Birgus* and *C. brevimanus* is effected by a behavioral rather than a physiological device, for the low blood concentrations cannot be maintained by either species in the absence of water of low salinity (Figs. 2 and 3). *C. perlatus* has demonstrated in the laboratory an ability to control its blood concentration by selectively entering sea water or fresh water (Gross and Holland, 1960). Yet this ability is not obvious in the field studies of this investigation. However, the paucity of fresh water or brackish water during the dry season probably precluded this behavioral activity. That is, tolerance to a wide range of blood osmotic concentrations by this species has permitted it to thrive in a variety of habitats, even though it probably is more selective when given the opportunity by the rainy season. Nevertheless, it is apparent that the dry season imposes no critical stress on the population of *C. perlatus*.

Birgus and *C. brevimanus* were usually confined to more specific environments than *C. perlatus*. Perhaps this precise discrimination is important in limiting the numbers of *C. brevimanus* and *Birgus* at Eniwetok, although both species show tolerance to considerable range of blood concentrations and *Birgus* on Jieroru Islet certainly demonstrated adaptability to stressed conditions.

Finally, it is suggested that the three subject species of this study represent three steps toward terrestrialness from the sea. *C. perlatus* seems closest to a marine existence because it habitually enters the sea to fill its adopted shell, a behavioral mechanism which was probably necessary for the initial step onto land by the hermit crabs; it prefers sea water to fresh water when offered a choice (Gross and Holland, 1960), and its high blood concentration in the field reflects an absence of fresh water which would be found only on land. Gibson-Hill (1947) considered that this species on Christmas Island is more strictly a marine animal which occasionally wanders away from the sea. *C. brevimanus* seems next closest to the sea. Although its blood concentration is dilute to sea water, it is more concentrated than that of *Birgus*. This species prefers fresh water to sea water when offered a choice, but it is still bound to an aquatic life by its adopted shell in which it carries a water supply of low salinity. *Birgus*, then, is most terrestrial, being independent of a gastropod shell, preferring fresh water to sea water and also having a blood concentration which is lowest of the three species when found in similar environmental situations.

Gross (1963b) has demonstrated that the terrestrial brachyuran crab, *Gecarcinus*, can tolerate sea water as its sole source of water only for about two weeks. This limited tolerance seems to be imposed by the inability of *Gecarcinus* to excrete salts. On the other hand, as mentioned above, *Birgus* can survive for at least 78 days on only sea water; yet after such treatment, its blood concentration (118% sea water), though higher than the available water, does not continue to elevate at the rate indicated in Figure 2. Rather, a plateau is reached at blood concentrations common to *C. perlatus* in nature, where the sea was its only source of water.

Thus, *Birgus*, which is probably the most terrestrial of the anomuran crabs, still seems closer to a marine existence or at least more tolerant of the sea than *Gecarcinus,* one of the most terrestrial of the brachyurans.

These studies were aided by a grant from the National Science Foundation, G-18978. The investigation at the Eniwetok Marine Biological Laboratory was supported financially by the U. S. Atomic Energy Commission. I wish to thank the following: Dr. Robert W. Hiatt for his role in making the Eniwetok trip possible; Dr. John S. Garth and Miss Janet Haig for kindly identifying *Coenobita brevimanus;* Dr. Edward Held for furnishing me unpublished information concerning the habits of *Birgus;* and Mr. Ronald Capen for his able technical assistance both in the field and in the laboratory.

SUMMARY

1. Water balance in the anomuran crabs, *Birgus latro, Coenobita perlatus* and *Coenobita brevimanus*, was studied in the field at Eniwetok Atoll during the dry season.

2. *Birgus* and *C. brevimanus* usually are found in wooded areas in or near piles of rotten cocoanuts which are damp in their interiors. The osmotic concentration of the serum of *Birgus* and *C. brevimanus* from such conditions was relatively constant, averaging 74.7 and 80.3% sea water, respectively. Such animals do not depend on the sea for their source of water.

3. The osmotic concentration of serum from *Birgus* captured on the exposed, dry islet of Jieroru averaged 91.6% sea water. Evidence suggests that these animals were either using the sea as their source of water or were tolerating slow desiccation in well-sealed burrows.

4. *Coenobita perlatus* is more common and less discriminating than the other two species in its choice of habitat. It is found in such extremes as exposed positions at the edge of the lagoon and protected conditions, such as the interior of piles of cocoanuts in wooded areas.

5. Serum osmotic concentrations for *C. perlatus* taken in the field were usually hypertonic to the available sea water and ranged from 102% sea water (cocoanut piles) to 150% sea water (active at night in forest).

6. *Coenobita perlatus* was observed to enter sea water and brackish water at night; this resulted in filling their adopted shells with water.

7. Neither *Birgus* nor *C. brevimanus* was observed in sea water in nature, although both species will use sea water under laboratory conditions.

8. Shell water found in *C. brevimanus* taken from cocoanut piles was always hypotonic to sea water, but essentially isotonic to the serum. The source of shell water in this species was not found, but experiments demonstrated that such large volumes are not secreted.

9. *C. brevimanus* can thrive on a damp substrate in the absence of surface water for more than two weeks. The serum concentration following this treatment remains relatively low (mean: 86.4% sea water), but abnormal quantities of fresh water and sea water are used by the animal following treatment. This suggests that dehydration occurred and that salts also were lost to the damp substrate.

10. The osmotic concentration of urine in all three species is close to that of the blood, although it tends to be slightly hypotonic to the blood in *C. brevimanus* and *Birgus*. Therefore, there is no evidence that the antennary glands of these crabs are especially adaptive for terrestrial life.

11. Evidence presented suggests that the three subject species represent three steps from a marine life toward a terrestrial existence, *Birgus* being considered the most terrestrial and *C. perlatus* the closest to the sea.

12. *Birgus*, probably the most terrestrial of the anomuran crabs, is physiologically more marine in the adult stages than *Gecarcinus lateralis*, one of the most terrestrial brachyuran crabs.

LITERATURE CITED

Arnow, T., 1954. The hydrology of the northern Marshall Islands. Atoll Research Bulletin No. 30, issued by The Pacific Science Board, National Academy of Sciences, National Research Council.

Bliss, D. E., 1956. Neurosecretion and the control of growth in a decapod Crustacean. *In:* Bertil Hanström: Zoological Papers in Honour of His Sixty-fifth Birthday, November 20th, 1956, ed. K. G. Wingstrand. Lund, Sweden: Zoological Institute.

Edney, E. B., 1960. Terrestrial adaptations. *In:* The Physiology of Crustacea, ed. T. H. Waterman, Vol. 1, 367–393. New York: Academic Press.

Gibson-Hill, C. A., 1947. Field notes on the terrestrial crabs. *Bull. Raffles Mus.*, **18**: 43–52.

Gross, W. J., 1955. Aspects of osmotic regulation in crabs showing the terrestrial habit. *Amer. Nat.*, **89**: 205–222.

Gross, W. J., 1963a. Acclimation to hypersaline water in a crab. *Comp. Biochem. Physiol.*, **9**: 181–188.

Gross, W. J., 1963b. Cation and water balance in crabs showing the terrestrial habit. *Physiol. Zöol.*, **36**: 312–324.

Gross, W. J., and P. Holland, 1960. Water and ionic regulation in a terrestrial hermit crab. *Physiol. Zöol.*, **33**: 21–28.

Harms, J. W., 1932. Die Realisation von Genen und die consecutive Adaption. II. *Birgus latro* L., als Landkrebs und seine Beziehungen zu den Coenobiten. *Zeitschr. f. wiss. Zool.*, **140**: 167–290.

Pearse, A. S., 1934. Freezing points of bloods of certain littoral and estuarine animals. *Carnegie Inst. Washington Papers from Tortugas Laboratory*, **28**: 93–102.

Prosser, C. L., J. W. Green and T. S. Chow, 1955. Ionic and osmotic concentrations in blood and urine of *Pachygrapsus crassipes* acclimated to different salinities. *Biol. Bull.*, **109**: 99–107.

Seurat, L. G., 1904. Observations biologiques sur les Coénobites (*Coenobita perlata,* Edwards). *Bull. Muséum d'Histoire Naturelle,* **10**: 238–242.

A STUDY OF THE MECHANISM OF PHOSPHATE TRANSPORT IN SEA URCHIN EGGS BY ION EXCHANGE ANALYSIS OF RAPIDLY LABELED COMPOUNDS[1]

W. M. GRIFFITHS AND A. H. WHITELEY

Department of Zoology and the Friday Harbor Laboratories, University of Washington, Seattle 5, Washington

The transport of phosphate into fertilized sea urchin eggs from sea water shows numerous characteristics of an enzymatically controlled transport, in which the mechanism is located in the cell surface. The reaction shows specificity for phosphate; the dependency of rate on phosphate concentration follows Michaelis-Menten kinetics; the reaction is competitively inhibited by arsenate and depends on surface-located sulfhydryl groups. It has a Q_{10} of 2.3, and shows a sharp pH optimum. The transport is unidirectional, carrying phosphate only inward. The transport itself shows little direct dependence on energy metabolism of the eggs. The mechanism is established as a consequence of fertilization (Abelson, 1947; Brooks and Chambers, 1948, 1954; Whiteley, 1949; Chambers and White, 1949, 1954; Lindberg, 1950; Villee and Villee, 1952; Chambers and Mende, 1953; Litchfield and Whiteley, 1959; Whiteley and Chambers, 1960, unpublished data).

To elucidate the mechanism and the process of its differentiation, the reaction catalyzed during the transport needs to be determined. The evidence available concerning the properties of the transport (Whiteley and Chambers, 1960, unpublished data) is compatible with the view that orthophosphate enters by phosphorolysis, perhaps as glucose-1-phosphate formed at the membrane. Lindberg (1950) reported that phosphate is incorporated into adenosine triphosphate at the egg surface. In red cells, it is suggested that phosphate is esterified as 1,3-diphosphoglycerate at the cell surface (Prankerd, 1956) or may be carried through the membrane as ATP[2] (Jonas and Gourley, 1954). Alternatively, a "carrier" leading to the transport of phosphate across the membrane in the form of unchanged orthophosphate can be envisaged, perhaps involving a permease mechanism as proposed for other transports by Rickenberg *et al.* (1956) for bacteria.

Information concerning the nature of the phosphate compound formed in the eggs during the transport would be helpful in clarifying this question. In an attempt to approach this goal, we have determined some of the chemical forms of phosphate that become labeled in fertilized sea urchin eggs during short exposures to radioactive orthophosphate. To increase the accumulation of the first formed compound, the metabolism of the eggs was inhibited by cyanide, azide or monoiodoacetate.

---

[1] This study was supported by a grant from the National Science Foundation to A. H. W.

[2] The following abbreviations have been used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; MIA, monoiodoacetate.

METHODS AND MATERIALS

The basic method used in all experiments involved the preparation of an acid-soluble extract of normal or inhibited fertilized sea urchin eggs after their exposure to radioactive orthophosphate, followed by the adsorption of phosphates on ion exchange columns and their differential elution and identification by the solvent system of Khym and Cohn (1953) and by paper chromatography. The radioactivity of the eluate was measured continuously with a ratemeter and the activity was recorded graphically.

Labeling of eggs with P^{32}. From 2.0 to 2.5 ml. of gently packed fertilized eggs, washed free of excess sperm, were cultured with gentle stirring at 13.0°–13.5° C. in 200–300 ml. of sea water at pH 8.0 for 100 minutes. At this time, when the phosphate uptake had reached its maximum rate (Whiteley and Chambers, 1960), the eggs were washed with fresh sea water, the volume reduced to 4.5–5.0 ml. and 50 or 100 μc. of P^{32} as orthophosphate were added. The eggs were rapidly agitated by swirling in 40-ml. conical centrifuge tubes at 13.5° C. for a predetermined period, after which they were diluted to 40 ml. with $-2°$ C. sea water in order to stop the uptake and metabolism of phosphate as completely as possible. The eggs were collected by centrifugation and washed 4–5 times with sea water at $-2°$ C., after which 3.0 ml. of iced 0.4 M perchloric acid were added. The washing at $-2°$ C. required 12–15 minutes. The final wash had negligible radioactivity.

In experiments with metabolic inhibitors, the cultured eggs were placed in 40 ml. of 5×10^{-3} M sodium azide, or 10^{-4} M potassium cyanide in sea water at pH 8.0, 13–15 minutes before adding P^{32} to give time for penetration of the inhibitor. Eggs were pretreated with 0.1 M sodium monoiodoacetate for 30 minutes. Control eggs of the same culture were left in sea water. The inhibitor was also added in the same concentration to the $-2°$ C. sea water in which the eggs were washed after labeling.

Preparation of acid-soluble extracts. The eggs, in 0.4 M perchloric acid, were homogenized, and the homogenates centrifuged 15 minutes at 20,000 g. The supernatant extracts were immediately neutralized to pH 7.0 with potassium hydroxide, the perchlorate precipitate removed, and the extract made to a known volume. An aliquot was assayed for total activity, and an appropriate volume used for partition of phosphate compounds by ion exchange chromatography. When a balance sheet of the activity in the acid-soluble and acid-insoluble fractions was desired, the precipitates were washed, and the centrifuged washes neutralized and added to the extract. All operations were performed near 0° C.

Ion exchange chromatography. A slight modification of the method described by Benson (1957) was used. Cations were replaced by hydrogen ions by passing the extract through a Dowex-50 cation exchange column, which was then washed with about 150 ml. of water until the wash was essentially free of radioactivity. The extract and wash were brought to pH 8.0 with ammonium hydroxide and put on a Dowex-1 anion exchange column. Seven eluting solutions (Table I) were used in place of the 9 in the original method, as preliminary experiments showed that very little of the activity was eluted by the two solutions omitted. Elution was at the rate of about 3 ml. per minute. Solutions and columns were maintained at 0° C.

Continuous recording of radioactivity of the eluate from the column. To obtain a direct graphical record of the chromatogram, the eluate was passed from the column through a small Plexiglas chamber (diameter 25 mm.; depth 3 mm.). The chamber had a thin window made of a piece of cleared 35 mm. Kodak film which was placed in contact with the end window of a Geiger-Müller tube. The radioactivity in the chamber was measured continuously with a rate-meter and recorded graphically by means of a strip chart recorder. Figure 1 is an example of a chromatogram obtained by this procedure. The different fractions were collected in flasks at 0° C.

Radioactivity measurements of the fractions. The total activities of the homogenates, the acid-soluble extracts, and the fractions collected from the columns were assayed on an aliquot of each by means of an end window Geiger-Müller scaler, counting the main samples to a minimum of 5000 counts.

Paper chromatography. While the phosphate compounds of each fraction from the Dowex-1 column can be in part identified from the data on authentic compounds supplied by Benson (1957), further identification of an unknown was made by cochromatography on paper with one or more known compounds of similar anion exchange characteristics. Where there was a very sharp peak of high activity, as in IX in Figure 1, a sample from the top of the peak was chromatographed directly. In other cases, the fraction was first lyophilized. The samples were treated with cation exchange resin to remove salts before application to the paper.

Descending paper chromatograms were run at room temperature using washed (1 N HCl, 2 N oxalic acid, distilled water) Whatman No. 1 paper, and the following solvent systems: (1) methanol:ammonium hydroxide:water (6:1:3) for two hours; (2) isobutyric acid:0.5 M ammonium hydroxide (10:6) for 12 hours. Phosphate compounds were located by spraying by the method of Hanes and Isherwood as modified by Bandurski and Axelrod (1951). Adenosine phosphates were also revealed by quenching of ultraviolet fluorescence.

Simplified scanning procedure for radioactive chromatograms. The position of a labeled unknown was determined by attaching the dried chromatogram by masking tape near the right edge of the chart of a Minneapolis-Honeywell Electronik strip chart recorder. The chart, geared to move 20 inches per hour, pulled the chromatogram in front of an end window Geiger-Müller tube clamped in position against the face plate of the chart. The tube was fitted with a brass cap provided with a slit 25 mm. long and 1.6 mm. or 6.6 mm. wide, depending on the level of radioactivity and the resolution needed. The Geiger-Müller tube fed a rate-meter, whose data were recorded dirctly on the strip chart beside the chromatogram. The tracings are in perfect register with the chromatogram, the procedure is simple, and no special equipment is needed. The tracings can be made quantitative by determining the area under the curve.

Ion exchange columns. Dowex-50 hydrogen (California Biochemical Corp., AG 50W-X8, 200–400 mesh) and Dowex-1 chloride (California Biochemical Corp., AG 1-X8, 200–400 mesh) resins were used in columns measuring 9.0 mm. × 100 mm. Both resins were freed of fines before using.

Arginine determinations were made by the method of Dubnoff (1957) on neutral or alkaline solutions containing 5–100 μg./ml. of arginine.

Living materials. Fertilized eggs of the sea urchin *Strongylocentrotus pur-*

puratus were used. The experiments were carried out from November to mid-July. For each experiment, gametes of a single female and male were used. Spawning was induced by electric shocks of 60 volts A. C. or by injection of 4.25% KCl. In the experiments to be reported, 95–100% of the eggs were fertilized, and development of controls through early pluteus larval stages was normal.

Chemicals. P^{32} as orthophosphate was obtained from Oak Ridge, Cat. No. P-1, and used as supplied. Sodium azide and sodium monoiodoacetate were obtained from Eastman Kodak Co.

RESULTS

Distribution of P^{32} in control eggs

1. *Total uptake and partition between acid-soluble and acid-insoluble fractions.* An appreciable amount of the added P^{32} was incorporated by the eggs during brief pulses. For control experiments this ranged from 6.4% to 39.1% during exposures of 10 seconds to 10 minutes. The experiments were not designed to obtain these uptake data with maximal accuracy, and the values are probably less than the maximum possible.

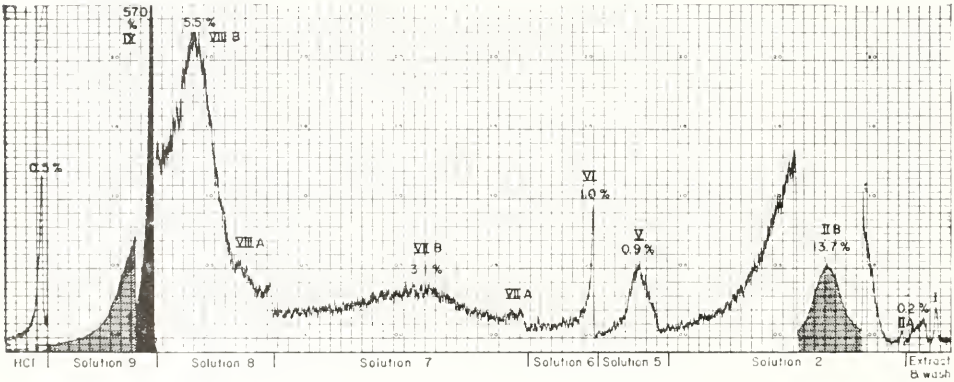


FIGURE 1. Graphical record of the radioactivity of the eluate from a Dowex-1 anion exchange column on which had been adsorbed the acid-soluble extract from control eggs exposed to P^{32} for one minute. The compositions of the eluting solutions, shown at the bottom, are given in Table I. The percentage of the acid-soluble activity is indicated above each peak. Full scale deflection equals 1000 c.p.m. (not hatched); 10,000 c.p.m. (hatched); or 25,000 c.p.m. (solid black).

In a few of the control experiments, partition of P^{32} between acid-soluble and acid-insoluble fractions was estimated by assaying the washed acid-insoluble residues in addition to the acid-soluble extract. Most of the activity recovered was in the acid-soluble fraction: 98.4% with a one-minute exposure to P^{32}, and 94.5% with a 10-minute exposure.

2. *Acid-soluble compounds bound by Dowex-50 cation exchange column.* When the neutralized extract was passed through a Dowex-50 column to remove cations, some of the radioactivity became bound at the top of the column and could not be removed by washing with water, although there was a continuous slight leakage of

activity, suggesting the presence of a labile compound. The active compound or its hydrolysis products could be eluted with 1.0 N HCl or 1.0 N NaOH. It is considered to be arginine phosphate on the basis of the following tests. Arginine phosphate is 98% hydrolyzed in 180 minutes at 37° C. in 0.01 N HCl (Mende and Chambers, 1953). A Dowex-50 column containing the unknown material was treated with two bed volumes of 0.01 N HCl, plugged and incubated at 37° C. for 180 minutes. After this, 87% of the activity, presumably due to inorganic phosphate released by hydrolysis, could be washed out of the column with water.

TABLE I

The distribution of P^{32} among the acid-soluble fractions from fertilized sea urchin eggs exposed to P^{32} for one minute. The fractions I–IX are shown graphically in Figure 1 and were eluted from an anion exchange column using the solutions 1–10 shown at the left of the table.

Eluting solution	Vol. ml.	Compounds eluted (Benson, 1957)	Fraction collected	% of total acid-sol. P^{32} activity	Radioactive compound identified
(1) Extract + H_2O wash of Dowex-50 column	150	—	I	0.2	—
(2) 0.025 *M* NH_4Cl + 0.01 *M* $Na_2B_4O_7$	1200	Glucose-1-P Pi	IIA IIB	trace 13.7	Pi Pi
(5) 0.03 *M* NH_4Cl	350	Glucose-6-P Fructose-6-P Ribose-5-P	V	0.9	Glucose-6-P
(6) 0.005 *M* HCl	360	AMP	VI	1.0	Unknown, not AMP
(7) 0.01 *M* HCl	1200	2-P-glycerate ADP	VIIA VIIB	trace 3.1	— ADP
(8) 0.02 *M* HCl + 0.02 *M* KCl	450	Fructose-di-P	VIIIA VIIIB	trace 5.5	— Unknown, not fructose-di-P
(9) 0.02 *M* HCl + 0.2 *M* KCl	450	ATP	IX	57.0	ATP
(10) 1.0 N HCl	150	—	X	0.5	—

Total from anion column	81.9%
Arginine-P from cation column	19.1%
Total recovery	101.0%

Repetition of the hydrolysis removed a further 11% of the activity. That only 87% was hydrolyzed in place of the expected 98% may be due to a lower effective acid concentration in our test. In any case, the presence of a very acid-labile compound was established. In addition, P^{32} and chemically determined arginine (Dubnoff, 1957) were eluted from a Dowex-50 column by 1.0 N NaOH in the same fractions and in roughly proportionate amounts. Lastly, arginine hydrochloride was adsorbed and eluted from Dowex-50 under the same conditions as was the labeled material.

In control experiments with periods of labeling of 10 seconds to 10 minutes, 9% to 27% of the P^{32} was incorporated into arginine phosphate.

3. *Acid-soluble compounds bound by Dowex-1 anion exchange column.* Figure 1 shows a typical record obtained from eluting a Dowex-1 anion exchange column on which had been adsorbed the labeled extract from control eggs exposed to P^{32} for one minute. The eluting solutions are shown in Table I, which also gives the amount of activity present in each fraction, expressed as a percentage of the total activity of the acid-soluble fraction, and summarizes the identifications of the fractions within the limitations of the observations so far made.

In Figure 1, peak I represents material, usually less than 0.5% of the total, that was not bound to the column due to overloading or too rapid application.

The two compounds known to be eluted by solution 2 are glucose-1-phosphate and inorganic phosphate. Peak IIB, which has 13.7% of the activity, is due to inorganic phosphate. Its elution from the column began after 50–100 ml. of solution 2 had passed through. Labeled inorganic phosphate, added by itself, is eluted similarly. In addition, when cochromatographed on paper with inorganic phosphate, using the two solvent systems described in methods, the radioactivity of the eluate and the added orthophosphate coincided. However, although strongly radioactive, there was insufficient inorganic phosphate present in the paper chromatogram to give any phosphate reaction when sprayed; the specific activity of the inorganic phosphate must be very high. Peak IIA, which was very small in this experiment, appeared a little earlier than the expected glucose-1-phosphate. The fraction was lyophilized and cochromatographed on paper with glucose-1-phosphate and inorganic phosphate. The small amount of radioactivity in the fraction coincided with inorganic phosphate, and not with glucose-1-phosphate. The latter was present in the extracts and gave a phosphate spray reaction on the paper, but had little or no label. A compound coinciding with glucose-1-phosphate was also present in the paper chromatogram of peak IIB. It was unlabeled.

Peak V is usually small, in this experiment comprising only 0.9% of the total activity. The compounds which are known to be eluted by solvent 5 are glucose-6-phosphate, fructose-6-phosphate, and ribose-5-phosphate. Cochromatography on paper of the lyophilized eluate showed that the radioactivity was not due to residual inorganic phosphate nor to fructose-6-phosphate. It coincided with glucose-6-phosphate.

Peak VI, which had 1.0% of the activity, is due to a substance with the elution characteristics of adenosine-5-phosphate. However, the ultraviolet absorption of the lyophilized fraction was inconclusive. Cochromatography on paper with adenosine-5-phosphate and inorganic phosphate showed that the radioactive compound was neither of these, nor was it residual glucose-6-phosphate from Peak V. From its ion exchange characteristics it should be a monophosphate.

Two substances are usually eluted with solution 7. The first of these, VIIA, is a minor component which might be 2-phosphoglycerate. The second component, with 3.1% of the total activity, has the elution characteristics of adenosine diphosphate and we have confirmed this by cochromatography.

Solution 8 is known to elute fructose-1, 6-diphosphate. In Figure 1 two minor unknown components, pooled in VIIIA, precede the major component, VIIIB, which accounts for 5.5% of the total activity. Paper chromatography of the lyophilized fraction VIIIB was difficult due to the high concentration of salts in the concentrated samples, but in no case did the radioactivity coincide with fructose-diphosphate nor is it due to inorganic phosphate hydrolyzed from the ATP still on the column. Though radioactive, the component was not revealed by the phosphate spray reagent, and presumably has a relatively high specific activity. The identity of this major component is therefore unknown, but it should be a diacidic substance.

Solution 9 elutes ATP. In this experiment, 57.0% of the activity was in fraction IX. The activity was due to ATP, as determined both by cochromatography, and by the ultraviolet absorption spectrum. Frequently a small peak appears on the trailing shoulder of the ATP fraction. This could include other nucleoside triphosphates, known to be eluted in this region (Cohn, 1957).

A residual 0.5% of the activity remained on the column. This was eluted with 1.0 N HCl.

Recovery from the columns was usually good. In this experiment, the total recovery was 101.0%, 19.1% being recovered from the Dowex-50 column and 81.9% from the Dowex-1 column.

In Table II data from this and other control experiments in which the eggs were exposed to P^{32} for one or 10 minutes have been tabulated. The partitioning of P^{32} among the fractions described is similar for both of these time intervals.

Effect of metabolic inhibitors on distribution of P^{32}

It has been shown previously (Litchfield and Whiteley, 1959; Whiteley and Chambers, 1960, unpublished data) that 5×10^{-5} *M* azide and 10^{-5} *M* cyanide have no effect upon the uptake of phosphate by the eggs although they have an inhibiting effect upon cleavage. In the present experiments with 5×10^{-3} *M* azide, 10^{-4} *M* cyanide and with 0.1 *M* monoiodoacetate, the extent of incorporation was as much as in the corresponding controls and in some cases more (Table II). Despite this strong uptake, these agents caused considerable changes in the distribution of P^{32} among the acid-soluble compounds after pulses of one or 10 minutes, as shown in Table II. In the presence of azide, Experiments 1 and 2, there is a great decrease in the percentage of label incorporated into the high energy phosphate compounds. In the 10-minute experiment, the ATP was reduced from 55.3% to 23.0%, and in the one-minute experiment, from 53.7% to 14.3%. The drop in arginine phosphate was nearly complete: from 11.9% to 0.3% and from 27.0% to 1.2%, respectively. A corresponding increase appeared in the labeled inorganic phosphate (the sum of IIA and IIB), which rose from 11.7% to 58.7% in the 10-minute P^{32} pulse, and from 7.7% to 76.9% in the one-minute pulse. There is thus a loss of 43.9% from the two high energy compounds and a gain of 47.0% in orthophosphate in the 10-minute experiment, and a 65.2% loss and 69.2% gain of these compounds in the one-minute experiment.

Very similar results were obtained using potassium cyanide as inhibitor. These are shown in Experiment 3 of Table II. Again there was a very great decrease in labeled high energy phosphate (arginine phosphate and ATP) and an equivalent increase in labeled inorganic phosphate. As with azide, the arginine phosphate fraction was essentially unlabeled.

TABLE II

The effect of metabolic inhibitors on the distribution of P^{32} among the acid-soluble fractions from fertilized sea urchin eggs exposed to P^{32}

	Duration of P^{32} pulse, minutes	% of P^{32} taken up by eggs	Eluted from Dowex-50 arginine-P %	Fractions eluted from Dowex-I column											Recovery %
				I %	IIA %	IIB Pi %	V Glucose-6-P %	VI %	VIIA %	VIIB ADP %	VIIIA %	VIIIB %	IX ATP %	1 N HCl strip %	
Exp. 1. Control	10	39.1	11.9	0.1	0.2	11.5	0.9	1.4	0.3	1.3	0.7	3.3	55.3	0.3	87.2
5×10^{-3} *M* NaN$_3$	10	47.8	0.3	0.2	0.5	58.2	1.0	1.7	trace	4.3	1.1	1.8	23.0	trace	92.1
Exp. 2. Control	1	6.4	27.0	0.4	0.9	6.8	0.8	0.8	trace	2.0	—	4.2	53.7	0.3	97.0
5×10^{-3} *M* NaN$_3$	1	9.7	1.2	0	0	76.9	1.9	1.2	0	4.2	—	3.9	14.3	0.2	104.8
Exp. 3. Control	1	29.3	19.1	0.2	trace	13.7	0.9	1.0	trace	3.1	trace	5.5	57.0	0.5	101.0
10^{-4} *M* KCN	1	42.1	0.6	trace	trace	71.7	1.8	1.2	trace	4.0	—	2.6	16.9	0.2	99.4
Exp. 4. Control	1	15.0	9.0	trace	2.3	16.1	5.7	0.4	trace	2.2	1.4	4.7	47.8	0.3	89.8
0.1 *M* MIA	1	14.9	9.0	trace	trace	35.0	3.3	0.5	trace	1.9	1.2	3.4	38.3	trace	92.6
Exp. 5. Control	1	24.5	12.5	0.9	0.8	6.1	5.9	1.3	trace	4.1	1.7	4.7	61.2	0.7	99.9

Changes in the other components are all small, but show the same pattern for both the azide and cyanide experiments. These include a moderate increase in label in glucose-6-phosphate, a small increase in fraction VI, a somewhat larger increase in ADP, and an appreciable drop in fraction VIII.

Both azide and cyanide caused the first cleavage of the eggs to be delayed by a length of time equal to or longer than the time of exposure. These inhibitions were largely reversible.

Less clear-cut changes in the distribution of P^{32} were obtained in the presence of 0.1 M monoiodoacetate (Table II). There was a reduction of 9.5% in ATP (38.3% in the experimental eggs compared with 47.8% in the controls) and an increase of 16.6% in inorganic phosphate (35.0% as compared with 18.4% for the controls). There was no change in the labeling of arginine phosphate. Both glucose-6-phosphate and fraction VIII were less strongly labeled in the presence of monoiodoacetate. A 30-minute exposure to the inhibitor caused a delay in the first cleavage of 14 minutes. On return to sea water subsequent development of these eggs was blocked in the gastrula stage.

Effect of length of P^{32} pulse on the distribution of P^{32} among the acid-soluble compounds

In an effort to establish the time sequence of labeling, the eggs were exposed to P^{32} for 10 minutes and for one minute in the control experiments described in Table II. It is clear that there is no consistent difference in the relative distribution of activity during these intervals. A technical weakness in these experiments was the relatively long time, about 10–15 minutes, required to wash the eggs free of all extracellular P^{32}. Although these washes were carried out in sea water at −2° C., undoubtedly some metabolism of phosphate continued during the washing period. In order to minimize this and so determine which phosphate compounds are labeled initially, experiments with 30-second and 10-second exposures to P^{32} were carried out with a modified procedure. At the end of these brief periods, the 5-ml. egg suspensions were diluted to 40.0 ml. with sea water at −2° C., centrifuged for 30 seconds at 500 g in the cold, the supernatant carefully removed, and perchloric acid added to the eggs without further washing. In this way, the time from the end of the P^{32} pulse to the addition of perchloric acid was reduced to two minutes at about 0° C. The supernatant sea water removed was assayed for P^{32} activity. The extracts were fractionated on the ion exchange columns and corrections were made on the assumption that 10% to 25% of the packed egg volume was due to extracellular fluid. The results of these experiments are in Table III, calculated on the basis that either 10% or 25% of the activity was due to extracellular radio-orthophosphate. The true value can be expected to lie between these extremes. The results are preliminary but are believed to approach the initial values more closely than do those from the one-minute and 10-minute experiments. They show again that more than half of the P^{32} is converted to high energy phosphates, even in these brief intervals, though the percentages are less than for the one- or 10-minute experiments. Further, there is a decided increase in the percentage of label in the orthophosphate fraction in the short-term experiments, reaching a value that may be as high as 39% in the 10-second experiment.

TABLE III

The distribution of P^{32} among the acid-soluble fractions from fertilized sea urchin eggs exposed to very short P^{32} pulses. Corrections have been made for P^{32} trapped in the intercellular spaces.

	Duration of P^{32} pulse, seconds	% of P^{32} taken up by eggs	Eluted from Dowex-50 arginine-P %	Fractions eluted from Dowex-1 column								Recovery %
				I %	IIA+B Pi %	V Glucose-6-P %	VI %	VIIA + B ADP %	VIIIA+B %	IX ATP %	1 N HCl strip %	
Exp. 6.												
Corrected for 10% intercellular space	10	18.4	12.0	1.3	39.2	0.3	0.6	1.0	2.7	39.7	0.2	97.0
Corrected for 25% intercellular space		17.1	14.9	1.6	24.8	0.4	0.7	1.2	3.3	49.0	0.3	96.2
Exp. 7.												
Corrected for 10% intercellular space	30	21.8	15.5	3.5	31.4	0.7	0.5	1.6	2.6	41.2	—	96.9
Corrected for 25% intercellular space		20.5	17.8	4.0	20.8	0.7	0.5	1.8	3.0	47.7	—	96.3

Discussion

Three different hypotheses have been advanced to explain the transport of substances across membranes where it can be shown that simple diffusion is not an adequate explanation. The first involves specific sites in the membrane where substances are either adsorbed to, or chemically combined with, a carrier molecule. The substance is liberated to the cytoplasm chemically unchanged. The second concept envisages the chemical change of the molecule at the cell surface during the transport, liberating it in a new chemical form to the cytoplasm. The third is that of pinocytosis. In the absence of evidence for pinocytosis in sea urchin eggs, this mechanism need not be considered here.

The present data may be considered with respect to the first two mechanisms. Identification of the first labeled form of phosphate, the transport form, would settle the question. The present biological material offers certain simplifications over other cells where phosphate transport has been studied: yeast (Rothstein, 1955), erythrocytes (Gourley, 1952; Prankerd, 1956), bacteria (Mitchell, 1959) and liver cells (Sacks, 1951). In sea urchin eggs the transport is strictly unidirectional and results in a very pronounced accumulation; it is not inhibited by cyanide, azide, monoiodoacetate, anaerobiosis, and 2,4-dinitrophenol (Litchfield and Whiteley, 1959; Whiteley and Chambers, 1960, unpublished data) nor does it require exogenous nutrients.

In other instances, precursor relationships among labeled phosphate compounds have been sought by determining relative specific activities, in order to establish the sequence in which phosphate passes through the membranes and into the metabolic pools. In the present study, the transport form of phosphate has been sought by taking advantage of the fact that the inhibitors do not block uptake, but do interfere with metabolic flow of phosphate. Thus, if an inhibitor does not inhibit the uptake but causes a great increase in label in one component and a decrease in others, then the latter compounds are unlikely candidates for the transport form, while the former compound is likely.

Lindberg (1950) proposed that phosphate penetration in sea urchin eggs was by formation of ATP near the surface. This mechanism was rendered improbable by the observations, cited above, that inhibitors of energy metabolism do not depress the rate of uptake of phosphate. The present observations substantiate that more than 50% of the radioactive phosphate is metabolized to ATP and arginine phosphate, even in 10-second pulses, and in the one- and 10-minute experiments the value is about 70%. But the fact that the inhibitors tested greatly depress the entrance of P^{32} into the high energy phosphates and increase the percentage in the inorganic phosphate pool is a strong argument against any mechanism involving the initial formation of any high energy phosphate compound.

The properties of the transport mechanism previously determined would permit the hypothesis that transport is by means of a surface-located glycogen phosphorylase which would phosphorylyze glycogen from the inside with phosphate from the outside, liberating glucose-1-phosphate into the cell. Such a mechanism has also been indicated as possible in phosphate transport in liver cells (Sacks, 1951). In this event, a high percentage of labeling of glucose-1-phosphate would be expected, but this was not found. In addition, the observed increase in percentage of P^{32} as inorganic phosphate and decrease in high energy forms in the presence of the inhibitors would not be expected. It is concluded that phosphate does not enter as glucose-1-phosphate.

In erythrocytes, Prankerd and Altman (1954) suggested that phosphate enters in the reaction, forming 1,3-diphosphoglycerate. In the sea urchin eggs, such a mechanism would account for the lack of label in glucose-1-phosphate and could account for the observations that azide increased the label in orthophosphate at the expense of ATP and arginine phosphate. According to Spiegelman *et al.* (1948), azide uncouples the oxidation of triosephosphate from the generation of ATP from 1,3-diphosphoglycerate. Labeled phosphate picked up at the membrane would thus be released in glycolysis as orthophosphate rather than as high energy phosphate. However, this explanation would not account for the similar results with cyanide, which has no such uncoupling effect. Further, monoiodoacetate, which inhibits triose dehydrogenase, would not cause the observed accumulation of label in the inorganic phosphate pool and should depress total uptake, especially if 1,3-diphosphoglycerate formation occurs at the surface. No such depression is observed.

For all these possible mechanisms, or any involving group-transferring reactions (Mitchell, 1959), one would expect the activity present in inorganic phosphate to increase with increased time of exposure to P^{32} as the label is cycled and released into the inorganic pool. On the contrary, the experiments indicate that the proportion of label present as inorganic phosphate to that as high energy phosphate is much higher in the short pulses than in the longer ones. In the 10-second experiment, this proportion is between 0.77 and 0.40 and in the one- and 10-minute experiments, it averages 0.15.

While the evidence of these experiments is opposed to the mechanisms so far described, they and the other known facts of phosphate transport in sea urchin eggs are not incompatible with a carrier molecule type of transport system, which would release inorganic phosphate unchanged into the metabolic pool in the eggs. The great increase in activity of the inorganic phosphate in metabolically blocked cells would be due to a continued high rate of pumping of inorganic radioactive phosphate and its greatly reduced use in oxidative phosphorylation to form ATP and arginine phosphate. The increased proportion of label appearing as inorganic phosphate as the pulse is shortened is in support of the idea that the transport form is orthophosphate. Such a carrier could have the specific properties of an enzyme, as, for example, the permeases of Rickenberg *et al.* (1956). The kinetic, specificity, and inhibition properties described by Whiteley and Chambers (unpublished data), and the highly polarized character of the transport are compatible with this suggestion. The negligible activity found in glucose-1-phosphate suggests that either a high concentration of this substance is present initially in the cell, and little is formed during exposure to P^{32}, or that the cytoplasm has at least two pools of inorganic phosphate, one of which receives labeled phosphate from the outside and supplies it to many metabolic reactions, and a second, unlabeled pool, which donates phosphate for the formation of glucose-1-phosphate. The carrier or permease model does not easily account for the observed lack of direct dependence of the transport on energy metabolism of the egg, particularly since accumulation takes place against a remarkable concentration gradient (Whiteley and Chambers, unpublished data). Conceivably, this transport has a high priority for the available energy mobilized in the cells in the presence of inhibitors.

SUMMARY

1. The mechanism involved in the transport of phosphate into fertilized sea urchin eggs has been studied by exposing eggs to pulses of P^{32} and examining the radioactive compounds formed. These have been identified in part by ion exchange and paper chromatography in control eggs and in the presence of metabolic inhibitors.

2. Control eggs of *Strongylocentrotus purpuratus* at the two-cell stage incorporated 6.4% to 39.1% of the P^{32} from sea water in 10 seconds to 10 minutes; most of this (98.4% for a one-minute pulse, and 94.5% for a 10-minute pulse) was in perchloric acid-soluble compounds.

3. The distribution of the label after one-minute and 10-minute pulses was similar. In a representative one-minute experiment 27.0% was found in arginine phosphate; 7.7% in inorganic phosphate; 0.8% in glucose-6-phosphate; 2.0% in ADP; 53.7% in ATP; and 5.7% was distributed among 5 unidentified fractions. Glucose-1-phosphate, while present in the extract, was without significant label.

4. In experiments in which one-minute and 10-minute pulses of P^{32} were given to eggs in the presence of 5×10^{-3} *M* sodium azide, or 10^{-4} *M* potassium cyanide, the incorporation of label was as much or more than in the controls, but its distribution was greatly altered, with similar changes for both azide and cyanide. In a one-minute pulse in the presence of azide, incorporation into arginine phosphate was reduced to 1.2% and into ATP to 14.3% while inorganic phosphate increased to 76.9%: a decrease of 65.2% in high energy phosphates and an increase of 69.2% in inorganic phosphate. Minor changes occurred in other fractions. Similar, but much less marked changes were produced by 0.1 *M* monoiodoacetate.

5. A modified procedure was used with very short P^{32} pulses of 10 and 30 seconds, in an attempt to determine the initially labeled compound. The shorter the pulse, the greater was the percentage incorporation into inorganic phosphate, with parallel decreases in arginine-phosphate and ATP.

6. The results are considered to be incompatible with any mechanism for transport involving the initial formation of high energy phosphate; or with transport as the result of surface phosphorolysis of glycogen to form glucose-1-phosphate; or with transport resulting from glycolytic esterifications. The results are compatible with a carrier type of transport which would release inorganic phosphate unchanged into the metabolic pool in the eggs.

LITERATURE CITED

ABELSON, P. H., 1947. Permeability of eggs of *Arbacia punctulata* to radioactive phosphorus. *Biol. Bull.*, **93**: 203.

BANDURSKI, R. S., AND B. AXELROD, 1951. The chromatographic identification of some biologically important phosphate esters. *J. Biol. Chem.*, **193**: 405–410.

BENSON, A. A., 1957. Sugar phosphates, paper and column chromatography. *In:* Methods in Enzymology III. Edited by S. P. Colowick and N. O. Kaplan; pp. 110–129. Academic Press, New York.

BROOKS, S. C., AND E. L. CHAMBERS, 1948. Penetration of radioactive phosphate into the eggs of *Strongylocentrotus purpuratus*, *S. franciscanus*, and *Urechis caupo*. *Biol. Bull.*, **95**: 262–263.

BROOKS, S. C., AND E. L. CHAMBERS, 1954. The penetration of radioactive phosphate into marine eggs. *Biol. Bull.*, **106**: 279–296.

Chambers, E. L., and T. J. Mende, 1953. Alterations of the inorganic phosphate and arginine phosphate content in sea urchin eggs following fertilization. *Exp. Cell Res.*, **5**: 508–519.

Chambers, E. L., and W. E. White, 1949. The accumulation of phosphate and evidence for synthesis of adenosine-triphosphate in the fertilized sea urchin egg. *Biol. Bull.*, **97**: 225–226.

Chambers, E. L., and W. E. White, 1954. The accumulation of phosphate by fertilized sea urchin eggs. *Biol. Bull.*, **106**: 297–307.

Cohn, W. E., 1957. Methods of isolation and characterization of mono- and polynucleotides by ion exchange chromatography. *In:* Methods in Enzymology III. Edited by S. P. Colowick and N. O. Kaplan; pp. 724–743. Academic Press, New York.

Dubnoff, J. W., 1957. Glycocyamine and creatine. *In:* Methods in Enzymology III. Edited by S. P. Colowick and N. O. Kaplan; pp. 635–639. Academic Press, New York.

Gourley, D. R. H., 1952. The role of adenosine triphosphate in the transport of phosphate in the human erythrocyte. *Arch. Biochem. Biophys.*, **40**: 1–12.

Jonas, H., and D. R. H. Gourley, 1954. Effect of adenosine triphosphate, magnesium and calcium on the phosphate uptake by rabbit erythrocytes. *Biochem. Biophys. Acta*, **14**: 335–345.

Khym, J. X., and W. E. Cohn, 1953. The separation of sugar phosphates by ion exchange with the use of the borate complex. *J. Amer. Chem. Soc.*, **75**: 1153–1156.

Lindberg, O., 1950. On surface reactions in the sea urchin egg. *Exp. Cell Res.*, **1**: 105–114.

Litchfield, J. B., and A. H. Whiteley, 1959. Studies on the mechanism of phosphate accumulation by sea urchin embryos. *Biol. Bull.*, **117**: 133–149.

Mende, T. J., and E. L. Chambers, 1953. The occurrence of arginine phosphate in echinoderm eggs. *Arch. Biochem. Biophys.*, **45**: 105–116.

Mitchell, P., 1959. Structure and function in microorganisms. *Biochem. Symp.*, **16**: 73–93.

Prankerd, T. A. J., 1956. The activity of enzymes in metabolism and transport in the red cell. *Int. Rev. Cytol.*, **5**: 279–301.

Prankerd, T. A. J., and K. I. Altman, 1954. A study of the metabolism of phosphorus in mammalian red cells. *Biochem. J.*, **58**: 622–633.

Rickenberg, H. V., G. N. Cohen, G. Buttin and J. Monod, 1956. La galactoside-perméase d'*Escherichia coli*. *Ann. Inst. Pasteur*, **91**: 829–857.

Rothstein, A., 1955. Relationship of the cell surface to electrolyte metabolism in yeast. *In:* Electrolytes in Biological Systems. Edited by A. M. Shanes; pp. 65–100 American Physiological Society, Washington, D. C.

Sacks, J., 1951. Phosphate transport and turnover in the liver. *Arch. Biochem.*, **30**: 423–427.

Spiegelman, S., M. D. Kamen and M. Sussman, 1948. Phosphate metabolism and the dissociation of anaerobic glycolysis from synthesis in the presence of sodium azide. *Arch. Biochem.*, **18**: 409–436.

Villee, C. A., and D. T. Villee, 1952. Studies on phosphorus metabolism in sea urchin embryos. *J. Cell. Comp. Physiol.*, **40**: 57–71.

Whiteley, A. H., 1949. The phosphorus compounds of sea urchin eggs and the uptake of radio-phosphate upon fertilization. *Amer. Nat.*, **83**: 249–267.

Whiteley, A. H., and E. L. Chambers, 1960. The differentiation of a phosphate transport mechanism in the fertilized egg of the sea urchin. *Symp. on Germ Cells and Development* (Institut. Intern. d'Embryologie and Fondazione A. Baselli) : 387–401.

ERYTHROCYTE MEASUREMENTS IN FISHES, AMPHIBIA, AND REPTILES[1]

F. A. HARTMAN AND M. A. LESSLER

Department of Physiology, The Ohio State University, Columbus 10, Ohio

Gulliver (1875) made an extensive study of the size of the red cells in vertebrates, extending over a period of years. Cullen (1903) gives values for the skate and dogfish, while Cleland and Johnston (1912) reported a considerable series, especially birds. Although Wintrobe (1961) tabulated the results of many investigators on red cell size, there have been relatively few reports of new red cell measurements and many species remain unreported.

The size and shape of red cells gives an indication of the surface available for the exchange of gases in respiratory functions. Study of erythrocytes in many different species provides an interesting comparison of red cell size in relation to activities and habits. While collecting specimens for adrenal studies, we were frequently able to make fresh blood smears from the animals. These were air-dried and later stained and studied. The material was obtained in Panamá and the United States.

MATERIALS AND METHODS

Blood smears were made immediately after the death of the animal. These were air-dried promptly and stored until prepared for study in the laboratory. Staining was carried out under controlled conditions. Five drops of standard Wright's stain were allowed to remain on the slide for one minute before addition of a pH 6.5 buffer. The slide was allowed to stand for five minutes at room temperature and then washed with distilled water for 30 seconds and allowed to dry.

Ten cells on each slide, selected for excellence of staining and internal cytology, were measured by means of a calibrated eyepiece used in conjunction with an oil-immersion objective. In this way ten measurements of maximum cell width and length, as well as nuclear width and length, were made. The ratios of cytosome length to width and nucleus length to width have been calculated. This is a measure of cell and nuclear deviation from the spherical shape.

Erythrocytes of eight species among seven families of fishes, five species among five families of Amphibia, and thirty-three species among seven families of reptiles were measured (Table I). When measurements were made of only one individual of a species, the standard error of values for the different cells is shown. When

---

[1] Fishes were collected at the Marine Biological Laboratory, Woods Hole, Massachusetts. Other material was obtained in Ohio; in Florida with the Archbold Biological Station as a base; and in Panamá under the auspices of the Smithsonian Institution. We are indebted to Richard Archbold for his assistance, to Dr. Carl Johnson for the use of the Juan Mina Field Station of the Gorgas Memorial Institute and to Alois Hartmann for facilities near Volcan in West Panamá.

TABLE I

Erythrocyte measurements in fishes

Family and species	Cytosome		Ratio L/W	Nucleus		Ratio L/W
	Length μ	Width μ		Length μ	Width μ	
Rajidae						
Raja erinacea	20.3 ±1.5	12.9±1	1.57	8.4± .8	6.1± .3	1.37
Dasyatidae						
Dasyatis centrura (4)	19.7 ± .38	13.8±0.24	1.42	8.1±0.24	6.9±0.24	1.17
Anguillidae						
Anguilla rostrata	11.5 ±0.3	7.6±0.3	1.51	3.8±0.2	3.2±0.5	1.19
Leptocephalus conger	10.9 +1.5	7.7±1	1.43	4.0±0.5	3.3±0.5	1.21
Labridae						
Tautogo onitis (3)	10.5 ±0.35	7.3±0.18	1.44	4.1±0.15	3.0±0.15	1.37
Triglidae						
Prionotus carolinus (4)	11.15±0.15	7.3±0.32	1.53	4.8+0.28	3.0±0.15	1.37
Batrachoididae						
Opsanus tau	14.9 ±2	12.9±2	1.15	5.8+0.8	5.2±1	1.11
Lophiidae						
Lophius piscatorius	11.6 ±2	10.3±1	1.13	5.1+0.5	4.6±0.5	1.11
	13.6 +1.5	11.2±1	1.19	5.3+0.8	4.7±1	1.13

three or more individual specimens of a species were measured, the standard error is based on the averages of the individuals involved.

RESULTS

Among the fishes studied, the elasmobranch erythrocytes are nearly twice the size of the erythrocytes in the teleosts, and their width is a little more than half their length. The cytosomes of *Opsanus* and *Lophius* are almost as wide as long (ratio 1.15 to 1.19). In all of the fishes the ratio of length to width of the nucleus

TABLE II

Erythrocyte measurements in Amphibia

Family and species	Cytosome		Ratio L/W	Nucleus		Ratio L/W
	Length μ	Width μ		Length μ	Width μ	
Amphiumidae						
Amphiuma tridactylum	65.3±1.27	36.6±0.39	1.78	26.4±0.76	16.8±0.45	1.57
Proteidae						
Necturus maculosus	56.4±0.77	38.1±0.76	1.48	30.2±0.85	17.4±0.32	1.74
Bufonidae						
Bufo marinus	18.5±1.04	12.5±0.30	1.48	7.4±0.56	5.2±0.46	1.42
	19.2±0.91	13.4+0.53	1.43	7.6±0.38	5.6±0.41	1.36
Hylidae						
Hyla gratiosa	20.3±0.22	13.4±0.27	1.52	7.4±0.17	4.5±0.15	1.64
Ranidae						
Rana catesbiana	27.9±0.45	15.4±0.40	1.81	10.3±0.31	5.6±0.27	1.84

shows little range (1.11 to 1.37), the width being somewhat less than the length.

The tailed Amphibia possess by far the largest erythrocytes observed in our study (56 μ to 65 μ by 37 μ to 38 μ) while the erythrocytes of the anurans is one-half this size or smaller (19 μ to 28 μ by 13 μ to 15 μ) (Table II). The ratio of length to width in the cytosomes is 1.5 or larger. The ratio of length to width in the nuclei is of similar range.

Among the reptiles, in the one species of crocodilids studied, the cytosome and nuclear measurements are similar to those in elasmobranchs except that the ratios of length to width in the cytosomes and nuclei are greater (Table III). In the lizards there are considerable differences. Reptilian red cell lengths range from 15 μ to 19 μ with widths from 7.5 μ to 12 μ. Their length to width ratios vary from 1.44 to 2.06. Likewise there is a great range between nuclear measurements (5.1 μ to 8 μ) and their length to width ratios are from 1.19 to 2.11.

TABLE III

Erythrocyte measurements in a crocodilian and in lizards

Family and species	Cytosome		Ratio L/W	Nucleus		Ratio L/W
	Length μ	Width μ		Length μ	Width μ	
Crocodilidae						
Cayman sclerops (3)	20.6±0.59	10.9±0.46	1.88	7.1±0.19	4.1±0.57	1.74
Iguanidae						
Anolis carolinensis	14.8±0.42	10.3±0.39	1.44	6.0±0.06	3.8±0.18	1.59
Anolis polylepis (3)	18.4±0.47	10.1±0.87	1.84	7.6±0.29	4.8±0.27	1.58
Anolis pachypus	15.6±0.83	8.3±0.44	1.88	6.1±0.75	3.0±0.17	2.03
Anolis biporcatus (3)	18.5±0.37	12.0±0.64	1.54	8.0±0.18	5.2±0.10	1.54
Sceloporus malachiticus (6)	16.9±0.44	9.8±0.27	1.72	6.6±0.25	3.5±0.23	1.89
Basiliscus americanus	19.3±1.04	11.6±0.57	1.60	7.6±0.42	6.4±0.40	1.19
	18.6±0.31	12.1±0.47	1.54	6.5±0.15	5.5±0.12	1.18
Iguana iguana	15.4±0.28	7.5±0.37	2.06	5.6±0.24	4.5±0.35	1.34
Teiidae						
Anadia ocellata	16.3±1.18	9.0±0.76	1.81	5.7±0.35	3.2±0.27	1.78
Cnemidophorus sexlineatus	15.8±0.32	9.3±0.20	1.70	6.4±0.15	3.0±0.12	2.11
Ameiva praesignis (3)	15.0±0.16	8.6+0.46	1.75	5.9±0.23	3.5±0.20	1.66
Scincidae						
Eumeces fasciatus	15.9±0.16	8.5±0.16	1.87	5.1±0.16	3.1±0.10	1.67

In general, erythrocytes of snakes are large (15.5 μ to 23 μ); some are even larger than those in elasmobranchs. The cytosome length to width ratios range from 1.2 to 1.86 and the nuclei length to width ratios from 1.35 to 2.5. Measurements in three species of *Bothrops* indicated that their erythrocytes are the largest of all reptiles studied (Table IV).

In order to permit free movement of erythrocytes we can assume that the diameter of the smallest capillaries must be no less than the smaller diameter of the erythrocyte. With this assumption it is interesting to compare the minimal capillary diameters of different species and groups of vertebrates. The capillaries of fishes range from 7.3 μ to 13.8 μ in diameter. The largest capillaries are found in the elasmobranchs and the smallest among the bony fishes. The capillaries of Amphibia would range from 12.5 μ to 13.4 μ, while those of reptiles would range from 7.5 μ to 13.8 μ. The smallest are found in the lizards.

Discussion

Cytosome size

Since the erythrocyte is the most important carrier of oxygen and carbon dioxide, its surface area to size ratio is a determining factor in the exchange of these gases with the tissues. Thus, a small corpuscle offers the possibility of a greater rate of exchange than a larger one. Likewise an elliptical body is more efficient than a spherical one of the same volume. Based strictly on geometrical considerations, the red cells of the bony fishes would be the most efficient and those of the tailed amphibians the least efficient. Compared with warm-blooded animals the erythrocytes of these poikilothermic animals would be much less efficient.

Table IV

Erythrocyte measurements in snakes and turtles

Family and species	Cytosome		Ratio L/W	Nucleus		Ratio L/W
	Length μ	Width μ		Length μ	Width μ	
Colubridae						
Spilotes pullatus	17.7±0.24	10.3±0.18	1.72	7.1±0.19	3.7±0.20	1.92
Dryadophis boddaerti	16.3±0.55	11.0±0.92	1.48	7.0±0.63	4.5±0.83	1.56
Masticophis flagellum (3)	16.5±0.23	12.6±0.49	1.31	6.8±0.12	4.2±0.11	1.62
Clelia clelia (3)	20.2±0.45	11.0±0.22	1.82	7.3±0.40	3.7±0.11	1.97
Erythrolamprus bizonus	19.8±0.34	13.0±0.27	1.52	8.0±0.27	3.7±0.09	2.16
Leimadophis epinephalus	16.9±0.94	11.7±0.52	1.44	7.7±0.45	3.6±0.33	2.14
Leimadophis taeniurus	19.2±0.46	12.3±0.13	1.56	7.8±0.34	3.1±0.13	2.52
Thalerophis occidentalis	15.5±0.20	11.7±0.28	1.32	6.0±0.59	4.2±0.32	1.43
Dendrophidion paucicarinatus	17.1±0.04	12.5±0.76	1.36	6.6±0.70	3.8±0.50	1.73
Thamnophis sauritus	16.4±0.23	13.4±0.27	1.22	7.4±0.17	4.5±0.15	1.64
Natrix sipendon	18.2±0.84	13.8±1.28	1.41	6.8±0.74	4.5±0.74	1.51
	18.4±0.35	12.3±0.25	1.49	7.4±0.24	4.3±0.09	1.72
Natrix taxispilota	18.5±0.28	11.7±0.26	1.58	5.8±0.11	4.3±0.12	1.35
Lampropeltis doliata	18.1±1.02	11.8±0.96	1.53	7.1±0.84	4.4±0.48	1.61
Elaphe guttata	18.9±0.28	11.8±0.16	1.59	6.2±0.14	3.6±0.13	1.72
Coluber constrictor (4)	15.3±0.11	12.1±0.34	1.26	6.7±0.28	3.8±0.08	1.76
Opheodrys aestivus	16.3±0.29	12.5±0.19	1.30	6.9±0.15	4.6±0.42	1.50
Viperidae						
Bothrops lateralis	22.2±0.39	11.9±0.17	1.86	5.9±0.12	4.0±0.07	1.47
Bothrops nummifer	23.4±1.01	13.6±0.78	1.72	7.5±0.25	4.8±0.50	1.56
Bothrops atrox	22.1±0.96	12.6±0.46	1.75	7.7±0.55	4.9±0.24	1.57
Testudinidae						
Gopherus polyphemus (3)	19.1±0.61	11.2±0.22	1.71	6.7±0.55	4.0±0.12	1.69
Pseudemys ornata	18.6±0.01	11.6±0.76	1.60	5.5±0.35	5.2±0.66	1.06

In mammals the non-nucleated disc-shaped red cells have diameters from 5 μ and 6 μ (horse, cow, pig, mouse and rat) to about 7.5 μ (chimpanzee, woodchuck and llama) (Wintrobe, 1961). In birds the nucleated elliptical erythrocytes have size ranges from 10.7 μ by 6.1 μ to 15.8 μ by 10.2 μ (Hartman and Lessler, 1963).

The red cell count in poikilotherms is low. Counts of 70,000 to 390,000 per mm.^{3} in elasmobranchs; 780,000 to 1,490,000 in bony fishes; 20,000 to 440,000 in Amphibia; 560,000 to 1,050,000 in reptiles have been reported. In homiotherms, the red cell count in birds ranges between 1,930,000 and 3,690,000 per mm.^{3} and in mammals it is much more variable, ranging from 6,300,000 per mm.^{3} in the chimpanzee to 18,000,000 per mm.^{3} in the llama (Wintrobe, 1961).

Cleland and Johnston (1912) measured the erythrocytes in Australian vertebrates. By far the largest erythrocytes they reported were found in the lung fish, *Ceratodus* (39 μ by 25 μ). The elasmobranch erythrocyte sizes they reported are similar to our measurements, as are the teleost erythrocytes, except those of *Terapion* which are quite small (6 μ to 7 μ by 6 μ to 7 μ). The erythrocytes of the lizards Cleland and Johnston measured showed a somewhat greater range in size than our measurements, but their other reptile erythrocyte measurements are similar to those of our specimens.

Nuclei

In all fish erythrocytes the nuclei are shorter in proportion to their width than are the cytosomes. In the amphibian erythrocyte nuclei there is usually little difference in these ratios. This is also true in one turtle and in *Cayman*. In lizards, six out of ten species possessed relatively rounder nuclei than cytosomes, while in snakes all but four species had nuclei longer than the cytosomes. Nuclei were relatively longer than the cytosomes in all but six species of birds, being shorter in four and doubtful in two (Hartman and Lessler, 1963). Longer nuclei could offer a somewhat greater surface for exchange with the cytoplasm. This may be a significant factor in nuclear-cytoplasmic exchange. Actually the role of the nucleus in red cell function of the many species with nucleated erythrocytes has never been determined.

SUMMARY

Measurements of erythrocytes and their nuclei were made in 8 species of fishes, 5 species of Amphibia, and 33 species of reptiles. The cytosomes of elasmobranchs are almost twice the size of those in teleosts, being 19 μ to 20 μ by 12 μ to 14 μ. The cytosomes of *Opsanus* and *Lophius* are almost as wide as long, while in other fishes they are much longer than wide. The cytosome measurements in the two anurans, *Hyla* and *Bufo,* are similar to those in elasmobranchs while those in *Rana* are larger. Cytosomes in the crocodile, turtles and some lizards are also similar in size to those in the elasmobranchs. The cytosomes of snakes are large (15.5 μ to 23 μ), those of *Bothrops* being the largest of all reptiles studied. Based on the assumption that the least diameter of the erythrocyte indicated the minimal diameter of the capillaries, the largest capillaries are found in the elasmobranchs and the smallest in the teleosts.

LITERATURE CITED

Cleland, J. B., and T. H. Johnston, 1912. Relative dimensions of red blood cells of vertebrates, especially birds. *Emu*, **11**: 188–197.

Cullen, E. K., 1903. Oval and round cells in blood of skate and dogfish. *Bull. Johns Hopkins Hosp.*, **14**: 352.

Gulliver, G., 1875. Observations on the size and shapes of the red corpuscles. *Proc. Zool. Soc. London*, **1875**: 474–495.

Hartman, F. A., and M. A. Lessler, 1963. Erythrocyte measurements in birds. *The Auk*, **80**: 467–473.

Wintrobe, M. M., 1961. Clinical Hematology. Lea and Febiger, Philadelphia.

DEVELOPMENT OF THE AMPHIBIAN EAR RUDIMENT IN EXPLANTS

HELEN WARTON KAAN[1]

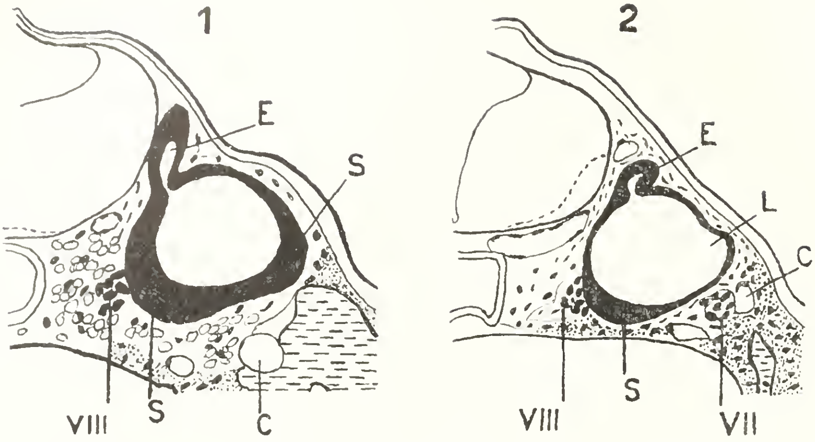
Marine Biological Laboratory, Woods Hole, Massachusetts

In connection with studies on the development of the cartilage capsule surrounding the auditory vesicle (Kaan, 1930), the ear rudiment of *Ambystoma maculatum* was transplanted into various regions of the young larva. These transplanted rudiments developed into relatively normal labyrinths only in the region immediately anterior to the normal ear. In other parts of the body, the transplants formed simple vesicles with or without sensory epithelium. The endolymphatic duct was absent and there was no indication of semicircular canals. Somewhat greater development occurred in ear rudiments of *Rana sylvatica* when transplanted into *Ambystoma* larvae. In later experiments (Kaan, 1938), dealing with the origin of the cartilage capsule, abnormalities occurred in the developing ear *in situ.* When the size of the ear region was reduced following the insertion of other tissues, the resulting labyrinth was reduced in size. When the capsule was defective, the corresponding portion of the labyrinth expanded into the available space, tending to form a cystic vesicle.

The occurrence of these developmental anomalies brought up the question to what extent the ear rudiment possessed an intrinsic capacity for differentiation, and it was, therefore, of interest to determine whether and how far it would develop when removed from the influence of the surrounding tissues and of the organism as a whole. Preliminary experiments indicated that an ear vesicle would develop in explants cultured in a sterile salt solution and consequently, two series of experiments were undertaken in the spring of 1945 at Stanford University on larvae of *Taricha rivularis* and *T. torosa.* Subsequent experiments were conducted in 1960 1961 and 1962 at the Marine Biological Laboratory on *Ambystoma maculatum* and, to a limited extent, on *A. tigrinum* and *Rana sylvatica.* Results of these experiments indicate that in all of the species the ear rudiment can develop in an explant enclosed wholly or partially by epithelium and accompanied by more or less of the mesendoderm and mesectoderm which normally surrounds it. Such an auditory vesicle can form an endolymphatic duct and areas of sensory epithelium and it is commonly associated with a ganglion, the eighth cranial (Yntema, 1937). The most advanced degree of differentiation attained by these explanted ear rudiments appears similar to that of normal salamander larvae at Stage 40 (Harrison) (Fig. 1) and of frog larvae at Stage 22 (Pollister and Moore, 1937) (Fig. 2). These stages represent the period of development when the individual cells of the labyrinth have utilized all of the nourishment within their own yolk granules and are dependent on material brought by the circulatory system. Differentiation of the

---

[1] Sarah Berliner Research Fellow of the American Association of University Women, 1944–1945, on leave of absence from Wellesley College, Wellesley, Massachusetts.



FIGURES 1 and 2. Normal left labyrinths of donor larvae.

FIGURE 1. EM-77, *Ambystoma maculatum*, Stage 40– (Harrison).

FIGURE 2. E$_s$-5, *Rana sylvatica*, Stage 22–23 (Pollister and Moore). These and the subsequent figures are tracings of camera lucida drawings at a magnification of 100 ×. They are represented semi-diagrammatically, with auditory vesicles in solid black, cartilage in fine stippling, muscle in short, horizontal lines; nuclei of cells are in solid black and yolk granules in open circles; inner and outer edges of surface epithelium are indicated by solid lines. E—Endolymphatic duct; S—Sensory epithelium; L—Pouch of lateral canal; C—Capillary; VII and VIII—Ganglia of seventh and eighth cranial nerves.

ear rudiment and of the surrounding tissues appear to be independent of each other except as developing endothelial sinuses may exert mechanical pressure within the confined space of the explant.

In recent years, the nomenclature of some species of salamanders has been changed and, since these are the animals most commonly used in embryological experiments, a certain amount of confusion of terminology exists in the literature. Throughout this paper, the author has used the nomenclature for each species as given by Gentry (1955) and Twitty (1959).

I wish at this time to express my sincere appreciation to Dr. Victor C. Twitty for his kindness in making the facilities of his laboratory available to me and for his suggestions concerning methods of technique.

MATERIAL AND METHODS

Two series of operations were performed on larvae of each of the five species. In the E Series, ectoderm with attached ear rudiment was cut from the right side of the head and an attempt was made to remove all of the mesendoderm from the inner surface of the excised piece, particularly those cells which were close to the ear rudiment. Varying amounts of neural crest were also removed. In the EM Series, both the mesendoderm and neural crest were left intact. Some variation occurred in the size of the piece of ectoderm but, in general, cuts were made in the hyomandibular groove and along the dorsal mid-line, with parallel cuts in the presomite and gill regions to include a piece of ectoderm approximately 0.6 mm. square. This provided enough ectoderm to completely enclose the ear rudiment and any surrounding tissues. Preliminary experiments had shown that degeneration occurred rapidly in explants with a small amount of ectoderm, leaving the ear rudiment exposed to the salt solution. Operations on the salamander larvae included Stages 23 through 32, with the majority at Stages 25 through 29. These represented development of the ear rudiment from the time just prior to formation of the placode through closure of the cup to form the otic vesicle. Frog larvae were operated on at Stages 16 and 17, corresponding to Stages 25 and 27 in the salamanders. Each experiment was continued as long as the explant appeared to be in a healthy condition. Table I summarizes the experiments in each series.

In 1962, 35 operations were performed on larvae of *A. maculatum* in which a portion of the lateral wall of the myelencephalon was included in the explant. Although several methods were used, the results were unsuccessful. The cut edges of the myelencephalon rolled outward, exposing the inner lining or the developing nerve cells and the ectoderm would not grow over this brain tissue. In most cases, the explant disintegrated rapidly; in others, either the piece of brain wall was healed out or the entire contents healed out, leaving a hollow ball of cells.

The sterile technique developed by Twitty was used in performing the operations and in the subsequent culture of the explants in modified Holtfreter's solution (Twitty, 1945). The operating instruments were finely-sharpened iridectomy scissors and steel needles. Both the donor larvae and the explant were transferred from the wax operating dishes, after 15–30 minutes, to an autoclaved glass stender dish and fresh salt solution. They were then left together in the same dish so that the experimental and control auditory vesicles would be subjected to the same

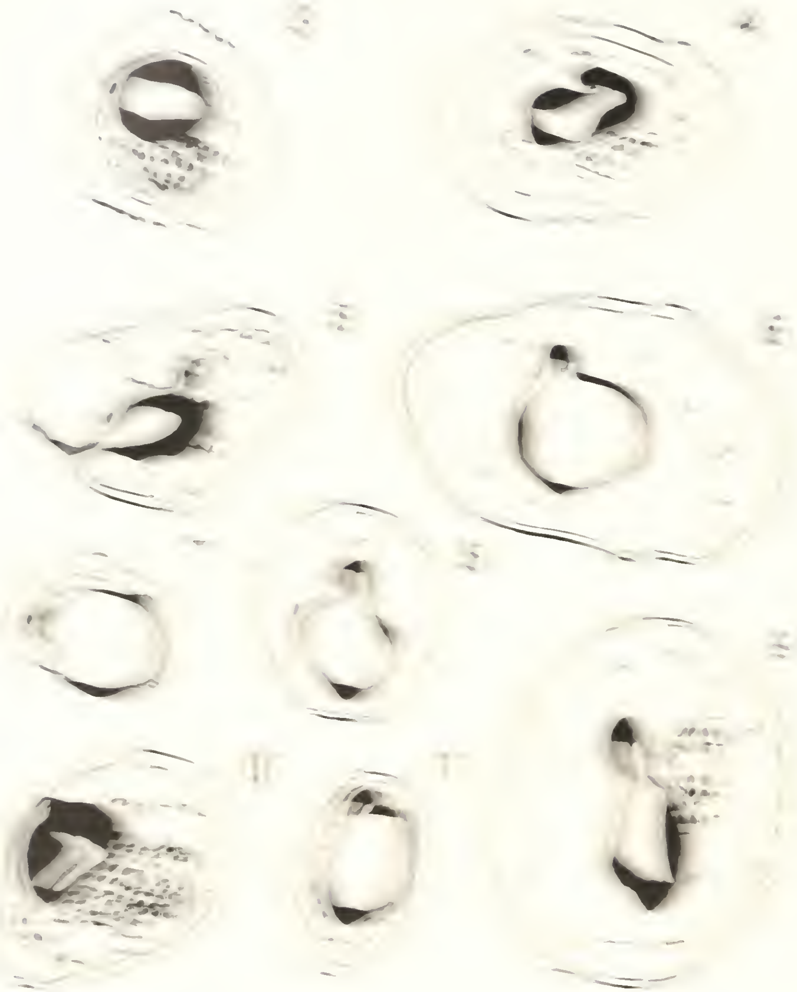
TABLE I

Summary of the E and EM series of explants in each of the five species

The individual species are distinguished as follows: E_r and EM_r—*Taricha rivularis*; E_t and EM_t—*T. torosa*; E and EM—*Ambystoma maculatum*; E_a and EM_a—*A. tigrinum*; E_s and EM_s—*Rana sylvatica*

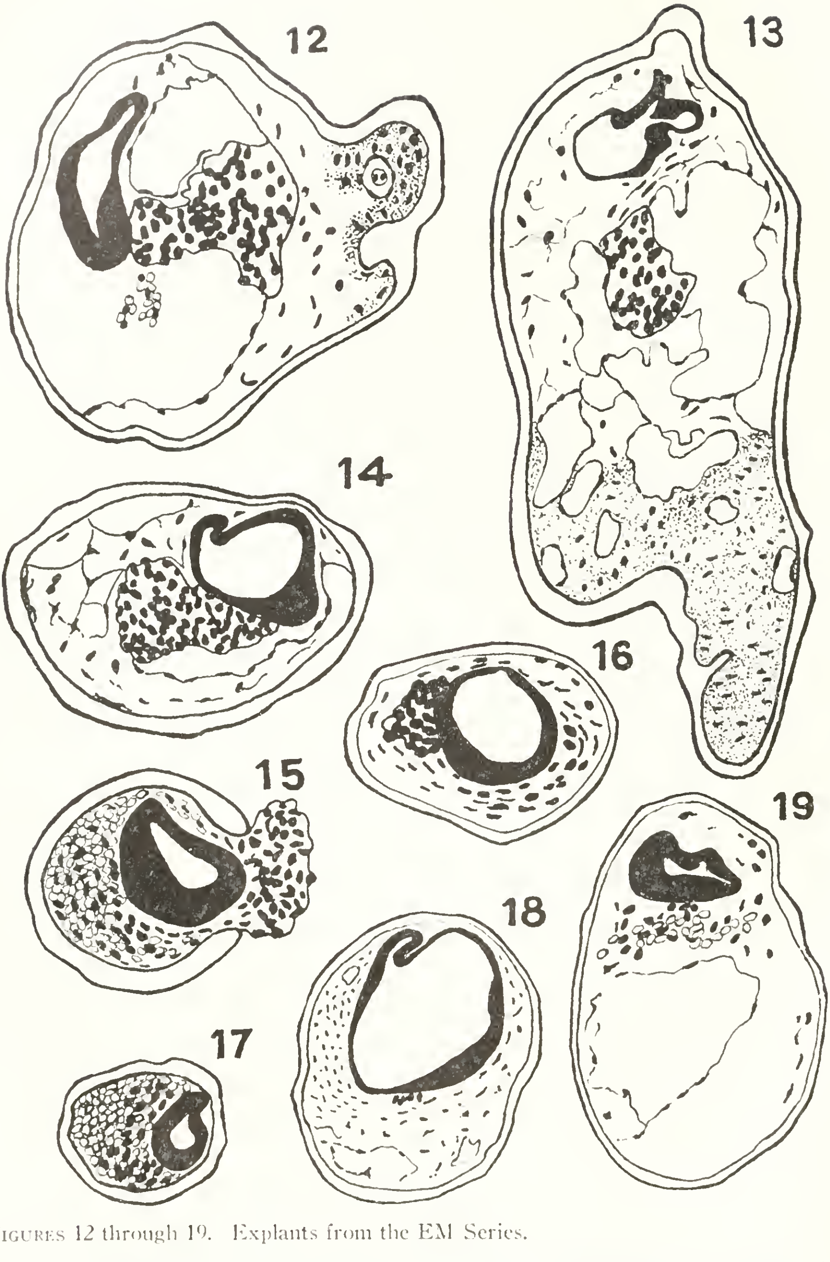
Series	Total no. of operations	Total no. of surviving explants	Number of explants studied	Stages at operation	Stages at preservation	Length of culture periods (no. of days)
E_r	36	28	25	24–29	37–45	15–26
E_t	58	40	31	24–32	41–45	12–24
E	37	12	10	24–29	39–42	6–12
E_a	10	8	7	25–29	33–38	3–6
E_s	6	3	3	17(27)*	22(40)*	4–6
EM_r	49	30	23	23–31	37–42	12–26
EM_t	48	37	28	24–31	40–45	14–26
EM	33	22	19	23–29	37–44	6–20
EM_a	10	7	6	25–29	34–36	2–8
EM_s	12	9	9	16–17 (25–27)*	20–23 (38–40+)*	3–6

* Figures in parentheses indicate the approximately comparable stage in salamander larvae.



[illegible]

[illegible]



FIGURES 12 through 19. Explants from the EM Series.

FIGURE 12. EM_l-22, *T. torosa;* operated at Stage 28; preserved after 14 days; larva at Stage 40. Vesicle is flattened by pressure of fluid in the large endothelial sinus but degree of differentiation is comparable to the normal left vesicle. Capillary within the rudimentary gill (right) contains a myelocyte.

FIGURE 13. EM_r-44, *T. rivularis;* operated at Stage 27; preserved after 26 days; larva not kept. Vesicle is compressed and distorted. Solid projection from upper part of vesicle is endolymphatic ducts and sensory areas could be identified (Figs. 5, 6, 7, 12, 14). Auditory vesicles of *T. rivularis* showed relatively little differentiation in explants of the E_r Series (Figs. 3, 4); it was difficult to determine either endolymphatic ducts or sensory areas. In vesicles of the EM_r Series, sensory areas were more distinct but endolymphatic ducts were very rudimentary or apparently absent (Fig. 13).

A number of exploratory operations, including both the E and EM types of explants, were performed on *A. maculatum* larvae prior to 1945. Since these operations, as well as the culture methods, were not standardized, only those experiments performed in 1960–1962 will be considered in this report (E-44 through E-81 and EM-47 through EM-80). Of these experiments, E-44 through E-63 and EM-47 through EM-76 were from eggs collected in the vicinity of Falmouth, Massachusetts; the remainder were from eggs shipped from Tennessee. There was no obvious difference in the development of larvae and explants from these two locations. There was also no clear indication that variations in the temperature at which the explants were cultured had any specific effect on the differentiation of the vesicles. Although the mortality was higher in the E Series, auditory vesicles in the surviving explants showed somewhat greater differentiation than those in the EM Series (Figs. 8, 9, 16). Figures 17 and 19 represent sections of two explants which developed from operations at stage 23, just prior to the appearance of the auditory placode. The vesicles are smaller than those from stages in which the ear rudiment is distinct but they show the beginnings of differentiation. Since another vesicle regenerated at the site from which the explant, EM-77, was removed, there is a question as to how much of the potential ear tissue was represented in the explanted vesicle. An interesting point which needs further investigation is that, in contrast to the explanted ear, the regenerated ear was very close in size and degree of differentiation to the unoperated right ear (Fig. 1).

Explants of *A. tigrinum* cannot be strictly compared with those of the other salamanders because their number was very limited and they were, of necessity, preserved at much younger stages (Stages 33–36). In both the E_a and EM_a groups, however, the degree of development of the explanted vesicles was directly

---

attached to surface epithelium in other sections. Endolymphatic duct cannot be definitely identified. Ganglion has only a slender attachment to vesicle. Section is through one of several "gills."

FIGURE 14. EM_t-8, *T. torosa;* operated at Stage 27; preserved after 18 days; larva at Stage 43. This is typical of explants in which there was no large accumulation of fluid. Pouch of lateral canal may have started.

FIGURE 15. EM_a-7, *A. tigrinum;* operated at Stage 27; preserved after 5 days; larva at Stage 35+. Vesicle is comparable to the left vesicle and shows the beginning of the endolymphatic duct and of sensory epithelium. The ganglion is not covered with ectoderm.

FIGURE 16. EM-55, *A. maculatum;* operated at Stage 29; preserved after 11 days; larva at Stage 40. This is typical of explants in this group. A rudimentary endolymphatic duct is present in other sections. Arrangement of cells around the vesicle suggests the beginning of capsule formation.

FIGURE 17. EM-77, *A. maculatum;* operated at Stage 23; preserved after 8 days; larva at Stage 40–. Normal left labyrinth shown in Figure 1. A second labyrinth regenerated on the right side at the site of the operation.

FIGURE 18. EM_s-7, *R. sylvatica;* operated at Stage 17+; preserved after 6 days; larva at Stage 23+ (Stage 3 of Birkman, 1940). Pouch of lateral canal is present. Vesicle is comparable to normal vesicle of Figure 2.

FIGURE 19. EM-80, *A. maculatum;* operated at Stage 23; preserved after 13 days; larva at Stage 41. Vesicle is compressed but shows greater differention than in EM-77.

comparable to that of the corresponding unoperated ears. The beginning sensory areas were indicated (Fig. 10) as well as the initial elongation which precedes the formation of the endolymphatic duct (Fig. 15).

Rana sylvatica larvae presented special conditions because their development was much faster than that of the salamanders. In less than one week, the auditory vesicles reached a degree of differentiation which required from two to three weeks in salamander larvae (Figs. 11, 18). It is interesting to note, however, that none of the explanted vesicles reached the same stage as the corresponding left vesicles which remained in the larvae. A comparison of Figure 11, showing the explanted right vesicle of E_s-5, with Figure 2, which represents the unoperated left vesicle of the same larva, brings out this difference.

Certain facts appear evident from a study of all of the explants: the explanted ear rudiment, when cultured in a balanced sterile salt solution, can differentiate to the extent of forming an endolymphatic duct and recognizable sensory areas; it can give rise to the eighth cranial ganglion; it does not develop beyond the equivalent of the salamander stage 40 or *Rana* stage 23, regardless of the length of the culture period and the degree of development of the ear in the donor larva.

The appearance and differentiation of tissues surrounding the explanted vesicles were of considerable interest, although they were not the primary concern of these experiments. The epithelium covering the explants varied from very thick, sometimes in solid masses, to a very thin, single layer over the distended areas. Typically, it consisted of the two layers of cells which are characteristic of the Amphibia and showed the same degree of development as the epithelium in the donor larvae. As the explant got older, an increasing number of cells in the outer epithelial layer became enlarged and vacuolated, presumably from the accumulation of fluid, and one of the signs of approaching disintegration of the explant was the rough appearance of the epithelium produced by these bulging cells. In the *Rana* explants suckers developed in the epithelium and produced mucus at about the same time as the suckers in the donor larvae.

Neural crest cells differentiated into sense organs of the lateral line system which could be distinguished in the surface epithelium (Stone, 1922). Pigment cells lying just beneath the epithelium undoubtedly arose from the neural crest as did the mesenchyme in some explants of the E Series (Figs. 6, 7, 8). Some of the mesenchyme may have come from mesendoderm cells which were not removed at the time of the operation. Presumably, mesendoderm gave rise, also, to the capillaries and endothelial sinuses of both series, as well as the muscle and blood cells which were present in certain explants of the EM Series. Origin of the cartilage which developed in some explants of the EM_r and EM_t groups (Figs. 12, 13) was not so obvious. However, it appeared only in the gill-like projections and this would indicate that it represented branchial arch material arising from mesectoderm (Stone, 1922). A careful study of all explants showed one in the EM group (Fig. 16), two in the EM_t group and two or three in the EM_s group (Fig. 18) in which the arrangement of cells next to the auditory vesicle suggested the formation of precartilage. This is particularly interesting in view of the fact that mesendoderm, the source of the auditory capsule (Stone, 1922, 1929; Mangold, 1937), was included in all explants of the EM Series.

It was apparent, then, that differentiation of the ear rudiments within the explants occurred independently of the surrounding tissues. These tissues did, however, influence the development of the ears to the extent that an epithelial covering prevented degeneration of the rudiment and that pressure from distended epithelial sinuses flattened or otherwise distorted the shape of the developing vesicle (Figs. 5, 9, 12, 13, 19). In the most extreme cases, ear rudiments were flattened against the surface epithelium and were unable to form vesicles.

Discussion

Stages 39 through 41 in the salamander and Stages 22–23 in the frog marked the apparent limit of development of the explanted ear rudiments. This period, just preceding the formation of the definitive labyrinth, is the time when yolk granules disappear from the individual cells of the auditory vesicle. The energy for further development, therefore, must come from sources outside the ear itself, since its intrinsic food supply has presumably been completely utilized. The fact that this period is a critical one has been brought out by investigations of several aspects of larval development.

Copenhaver (1926) observed completion of circulation in the gills of *A. maculatum* and an increase in rate of the heartbeat at Stages 36–37. A further increase in heart rate occurred at Stages 40–41. Pollister and Moore (1937) noted circulation in the gills of *R. sylvatica* at a comparable stage (Stage 20). In a comparison of *A. maculatum* and *A. tigrinum*, Hopkins and Handford (1943) recorded a gradual rise in oxygen consumption in both species up to Stage 37, with a more rapid rate of increase at the beginning of heart beat and muscular movement. The rate in *A. tigrinum* was higher than that in *A. maculatum.* Connon (1947) showed that, at a temperature of 20°, the respiratory rate of *T. torosa* was consistently higher than that of *T. rivularis* and exhibited a greater and more rapid increase. The curves in both species increased gradually during embryonic and early larval stages and more rapidly during later stages. He believed that there was a positive correlation between the developmental and respiratory rates, and noted that the yolk was used up more rapidly in *T. torosa.* Løvtrup (1953), using eggs, embryos and larvae of the axolotl (*A. mexicanum*), made a series of determinations of the utilization of carbohydrate, fat and protein at the different stages of development. He found that rate of carbohydrate consumption reached its peak during the neurula stages and then began to decline. Fat consumption began during the neurula stages and its rate increased during the larval stages. Thus, beginning with Stage 36, the rate of carbohydrate consumption was decreasing and the rate of fat consumption was increasing. Yolk, included within platelets or granules in the individual cells, contained a phospholipoprotein but no reducing carbohydrate. Using phosphoprotein phosphorus as an indication of the presence of yolk, he recorded a continual decrease, beginning soon after Stage 20. In a later, more detailed study (Løvtrup, 1955), he measured the activity of a series of enzymes, as well as RNA and DNA, in the embryos and larvae of *A. mexicanum*, *A. maculatum*, *R. platyrrhinus* and *Xenopus laevis.* He found that, for several of the substances tested, there was a steep rise in activity at about Stage 40 in *A. mexicanum* and *A. maculatum* and at Stages 21–23 in *R. platyrrhinus.* He concluded that this rise, coupled with the decrease in phosphoprotein phosphorus, indicated the end of the yolk reserve and the beginning of synthesis of new substances. This increased activity was correlated with the differentiation of liver and intestine, and the establishment of the vitelline circulation. Somewhat in the nature of a corollary to Løvtrup's experiments were those of Eyal-Giladi and Eyal (1962). They placed eggs and larvae of the axolotl in varying concentrations of chlorpromazine for varying lengths of time and determined the effects on development after removal to a solution of sulfadiazine and streptomycin. Among a variety of actions, chlorpromazine causes retardation of growth without malformations and the authors found that, under certain conditions, chlorpromazine permitted development up to Stages 37–38, followed by cessation of development and degeneration. They considered that these were critical stages with respect to oxygen consumption and, thus, particularly susceptible to the action of chlorpromazine.

Results of the foregoing experiments may be applied directly to the results obtained with the explanted ear rudiments. The most advanced stages of development reached by these auditory vesicles represent the limits of differentiation which can be attained through utilization of the yolk material in the individual cells. In normal larvae at comparable stages, there is an increase in respiratory rate and in oxygen consumption; changes in the nature of the food supply following depletion of the yolk involve the synthesis of new substances by enzymes in the developing digestive tract. The auditory vesicle is, therefore, dependent upon sources outside itself for the increased oxygen and food requirements necessary for further development and differentiation. At this time, the accelerated rate of the heartbeat and the establishment of the early circulatory system afford the means by which this is accomplished in the larva. Replacement of the salt solution by an adequate nutrient solution would be necessary to provide suitable conditions for further ear development in explants.

Detwiler and Van Dyke (1950) concluded that the results of transplantation experiments supported their view that ear rudiments normally depended upon the presence of the medulla for their differentiation. Confirmation of these results was not possible in the present series of experiments, since no explants were obtained in which a portion of the medulla was included. However, Mangold (1937) did succeed in getting viable explants from *Triton alpestris*, *T. taeniatus* and *A. mexicanum* which contained some brain tissue. These were all taken from neurula stages, prior to closure of the neural folds and formation of a definite ear rudiment. Otic vesicles appeared in some of the explants and, although none of them was complete, some did show endolymphatic duct, sensory areas, ganglion and the beginnings of definitive canals. Mangold believed that the presence of the medullary folds was a necessary and determining factor in the first appearance of the ear rudiment, but he was surprised that complete labyrinths did not develop, since the spatial conditions in the explant were favorable and the normal adjacent tissues were present. The present series of explants, containing a distinct ear rudiment, show that an auditory vesicle with endolymphatic duct, sensory areas and ganglion can be produced in the absence of medullary tissue and with minimum of other surrounding tissues, if the developing ear is enclosed by ectodermal epithelium. Further investigation is needed to determine definitely whether the formation of a complete auditory labyrinth is dependent upon the presence of the myelencephalon, the development of a cartilage capsule from the surrounding mesendoderm, or whether the ear is capable of still further independent development and differentiation in the presence of specific and essential nutrients.

In conclusion, then, it can be stated that explants of the ear rudiments of *T. rivularis, T. torosa, A. maculatum, A. tigrinum* and *R. sylvatica* are capable of undergoing development and differentiation when cultured in a sterile balanced salt solution. It appears to be essential that they be covered by ectoderm but their development is fundamentally independent of other surrounding tissues except as these exert mechanical pressure on the vesicle. Variations in temperature have no obvious effect on either mortality of the explant or development of the ear. Under these conditions, the ear rudiment may form a vesicle with endolymphatic duct and sensory epithelium and, in some cases, the beginning of the lateral pouch and separation of the sensory epithelium into two or more areas. The degree of differentiation of such a vesicle corresponds to about Stage 40 in salamander larvae and Stage 22 in the frog, a period in larval development when the individual cells of the ear have used up all of their intrinsic food material and are dependent on extrinsic sources for the nutrients needed in further development and differentiation. This does not necessarily represent the full capacity of the ear for independent differentiation. The exact nutritional requirements for expressing its ultimate potentiality may be determined through further experimentation with nutrient culture media.

SUMMARY

1. Two series of operations were performed on larvae of *Taricha rivularis, T. torosa, Ambystoma maculatum, A. tigrinum* and *Rana sylvatica*. In the E Series, a piece of ectoderm with attached ear rudiment was excised, and the mesendoderm and some, if not all, of the mesectoderm were removed. In the EM Series, mesendoderm and mesectoderm were included with the ear rudiment and ectoderm. These excised tissues were cultured, together with the donor larvae, in sterile Holtfreter's solution for varying lengths of time and, in most cases, at 15° or 16° C.

2. Study of serial sections of the explants showed that the ear rudiments of *T. rivularis, T. torosa, A. maculatum* and *R. sylvatica* were capable of independent development and differentiation up to the stage when the auditory vesicle possesses a distinct endolymphatic duct and sensory epithelium. This corresponds to Stage 40 in the salamanders and Stage 22 in the frog. In a few cases, there was some indication of the beginning of formation of a lateral pouch and separation of two areas of sensory epithelium. The explants of *A. tigrinum* were cultured for only a short period and consequently showed only the earliest signs of differentiation.

3. At Stages 40 (salamander) and 22 (frog), the cells of the auditory vesicles no longer contain visible yolk granules. They have, presumably, used up all of their intrinsic food material and are dependent on the developing circulatory and digestive systems for the supply of further nutrients.

4. A review of the literature indicates that these stages in the developing amphibian larva are marked by an increase in the heart rate and the rate of oxygen consumption, as well as an increase in the activity of specific enzymes which

synthesize new food materials to replace those originally present in the yolk granules.

5. It is concluded that the degree of differentiation attained by the ear rudiment when cultured as an explant in a balanced salt solution may not necessarily represent the limit of its potentiality for independent development. Further development might be possible if the explant were cultured in an adequate nutrient solution.

LITERATURE CITED

Birkman, K., 1940. Morphologisch-anatomische Untersuchungen zur Entwicklung des häutigen Labyrinthes der Amphibien. *Zeitschr. f. Anat. und Ent.*, **110**: 443–488.

Connon, F. E., 1947. A comparative study of the respiration of normal and hybrid *Triturus* embryos and larvae. *J. Exp. Zool.*, **105**: 1–24.

Copenhaver, W. M., 1926. Experiments on the development of the heart of *Amblystoma punctatum*. *J. Exp. Zool.*, **43**: 321–371.

Detwiler, S. R., and R. H. Van Dyke, 1950. The role of the medulla in the differentiation of the otic vesicle. *J. Exp. Zool.*, **113**: 179–199.

Eyal-Giladi, H., and Z. Eyal, 1962. The effect of chlorpromazine on the embryonic development of the Axolotl (*Ambystoma mexicanum*). *J. Emb.* and *Exp. Morph.*, **10**: 357–372.

Gentry, G., 1955. An annotated check list of the amphibians and reptiles of Tennessee. *J. Tenn. Acad. Sci.*, **30**: 168–251.

Hopkins, H. S., and S. W. Handford, 1943. Respiratory metabolism during development in two species of *Amblystoma*. *J. Exp. Zool.*, **93**: 403–414.

Kaan, H. W., 1930. The relation of the developing auditory vesicle to the formation of the cartilage capsule in *Amblystoma*. *J. Exp. Zool.*, **55**: 263–291.

Kaan, H. W., 1938. Further studies on the auditory vesicle and cartilaginous capsule of *Amblystoma punctatum*. *J. Exp. Zool.*, **78**: 159–183.

Løvtrup, S., 1953. Energy sources of amphibian embryogenesis. *C. R. Lab. Carlsberg. Sér. chim.*, **28**: 371–399.

Løvtrup, S., 1955. Chemical differentiation during amphibian embryogenesis. *C. R. Lab. Carlsberg. Sér. chim.*, **29**: 261–314.

Mangold, O., 1937. Isolationsversuche zur Analyse der Entwicklung der Gehör-, Kiemen- und Extremitätenregionen bei Urodelen. *Acta Soc. pro Fauna et Flora Fenn.*, **60**: 3–44.

Pollister, A. W., and J. A. Moore, 1937. Tables for the normal development of *Rana sylvatica*. *Anat. Rec.*, **68**: 489–496.

Stone, L. S., 1922. Experiments on the development of the cranial ganglia and the lateral line sense organs in *Amblystoma punctatum*. *J. Exp. Zool.*, **35**: 421–496.

Stone, L. S., 1929. Experiments showing the role of migrating neural crest (mesectoderm) in the formation of head skeleton and loose connective tissue in *Rana palustris*. *Arch. f. Entw.*, **118**: 40–77.

Twitty, V. C., 1945. The developmental analysis of specific pigment patterns. *J. Exp. Zool.*, **100**: 141–178.

Twitty, V. C., 1959. Migration and speciation in newts. *Science*, **130**: 1735–1743.

Yntema, C. L., 1937. An experimental study of the origin of the cells which constitute the VIIth and VIIIth cranial ganglia and nerves in the embryo of *Amblystoma punctatum*. *J. Exp. Zool.*, **75**: 75–101.

EVIDENCE FOR THE EXISTENCE OF HOMOZYGOUS CLONES IN THE SELF-FERTILIZING HERMAPHRODITIC TELEOST RIVULUS MARMORATUS (POEY)[1]

KLAUS D. KALLMAN AND ROBERT W. HARRINGTON, JR.

Genetics Laboratory, New York Aquarium, New York Zoological Society, American Museum of Natural History, New York 24, N. Y., and Entomological Research Center, Florida State Board of Health, Vero Beach, Florida

The prevalence of sexual reproduction among animals and plants attests to the great adaptive value of this mechanism. Members of sexually reproducing populations share a common gene pool from which a virtually unlimited number of genetic recombinations can be obtained through cross-fertilization, thus achieving the genetic variability that in the long run enables species to survive environmental change. There is also evidence that in Mendelian populations the heterozygous condition is superior in fitness to the homozygous one, heterozygous individuals showing greater adaptability to environmental variables.

Uniparental reproduction, on the other hand, prevents a species or race from acquiring new genotypes through recombination, and mechanisms such as parthenogenesis, gynogenesis and self-fertilizing hermaphroditism have been considered to lead into evolutionary blind alleys. Uniparental reproduction, to be sure, may be of temporary advantage in that favorable gene combinations can be maintained and rapidly passed on to future generations. All races and species that normally reproduce by these three mechanisms are composed of clones, the members of which have identical genotypes. In ameiotic parthenogenesis, the eggs do not undergo reduction divisions and the individuals arising from them are heterozygous, the degree of heterozygosity steadily increasing as gene and chromosome mutations accumulate. If this goes to the extreme, the two sets of chromosomes eventually become so dissimilar that the genome can no longer be considered diploid (White, 1954). In meiotic parthenogenesis the offspring may have arisen from haploid eggs, the diploid condition being reestablished either by the fusion of the egg nucleus with the second polar body nucleus or by the suppression of the first cleavage division. The first pathway leads to homozygosity within a few generations while the latter results in complete homozygosity in a single step. Hermaphroditism, when coupled with self-fertilization, results in a sharp decline in heterozygosity, leading to a population of homozygous individuals within 7 to 10 generations—in fact it constitutes the ultimate mode of inbreeding.

Several types of parthenogenesis are of regular occurrence in certain groups of invertebrates (Suomalainen, 1962; White, 1954), but well-documented cases of uniparental reproduction in vertebrates are extremely rare and each instance

---

[1] This investigation was supported by a research grant, CA 06665-01, of the National Cancer Institute, by a postdoctoral fellowship, HF-9500, of the U. S. Public Health Service to one of us (K. D. K.), and by research grant CC-00101-01 of the Communicable Disease Center, U. S. Public Health Service, to the junior author.

deserves special attention. A few teratological cases of self-fertilization resulting in uniparental offspring have been described in the guppy, *Lebistes reticulatus* (Spurway, 1957; Comfort, 1961), and Kilby and Kallman (unpublished) observed several cases of parthenogenesis in the mosquito fish, *Gambusia affinis*. Parthenogenesis has been found sporadically in a race of the domesticated turkey (for summary see Olsen, 1962).

Parthenogenesis occurs normally in several all-female subspecies of the Armenian lizard, *Lacerta saxicola* (Darewski and Kulikowa, 1961), and is suspected to occur in several other species of lizards in the genus *Cnemidophorus* (Maslin, 1962). In fishes, Hubbs and Hubbs (1932, 1946) discovered that *Mollienesia formosa* exists only as females, which reproduce by gynogenesis following insemination by males of related species. The sperm merely activates the eggs without contributing any genetic material. Gynogenesis has also been reported to occur in certain populations of the cyprinid *Carassius auratus gibelio* (Lieder, 1955).

Confusion exists in the literature with respect to the occurrence of hermaphroditism in fishes. In contrast to the assertions found in many recent text books and reviews, that the Sparidae are functional hermaphrodites, Reinboth (1962) points out that in the order Perciformes simultaneous functional hermaphroditism is restricted to certain species of serranids, protogyny occurs in some serranids, sparids, centracanthids and labrids while protandry is found in certain sparids. Among normally hermaphroditic perciforms, only in serranids are ovarian and testicular portions of the gonad active simultaneously. The eggs and sperm are emitted through separate ducts, and self-fertilization has been obtained experimentally in *Serranus scriba* (Reinboth, 1962; Salekhova, 1963) and in *S. subligarius* (Clark, 1959). Nevertheless, observations by all three investigators, in large-sized aquaria or in the natural habitat, indicate that spawning activity is initiated between two or more individuals, and it seems likely that cross-fertilization is the rule.

With the recent discovery of *Rivulus marmoratus* (Poey) along the Florida East Coast (Harrington and Rivas, 1958) and the subsequent finding that all collected specimens tested and most of their known descendants were functional, consistently self-fertilizing, oviparous hermaphrodites (Harrington, 1961, 1963), the possible homozygosity of these hermaphrodites and of their uniparental descendants came into question. The present report concerns the unique genetic relationship existing between normal self-fertilizing parents and their offspring. The tissue transplantation test (Kallman, 1962) has been used to determine whether parent and offspring have identical genotypes.

MATERIAL AND METHODS

Six self-fertilizing hermaphroditic *R. marmoratus*, collected in 1961 in their natural habitat near Vero Beach, Florida, and their descendants were used in the experiments described below. From the time of their capture the wild fish were kept isolated in small aquaria, and all future generations originated from individuals raised *ab ovo* in individual glass jars. Each egg thus allocated to its own rearing jar was obtained at the precise moment it was emitted (oviposited) by its hermaphrodite parent (for details, see Harrington, 1963). The six progenitors were designated *FT*, *NA*, *DS*, *NL*, *NSU* and *NSB*.

For ready identification of individual fish the following system has been adopted. The first letter following the hyphen always identifies fish of the first laboratory generation, the second and third letters identify the fish of the second and third generations, respectively. Thus *DS-ACH* is a third generation fish, "H," the offspring of second generation fish "C" which in turn was derived from fish "A" of the F_1 generation. The original progenitor was *DS*.

Anal, dorsal and caudal fins, hearts and spleens were transplanted according to a method described previously (Kallman and Gordon, 1958; Kallman, 1960). These structures were selected, because they can easily be grafted and their fate readily ascertained. Inadequate numbers of fish in certain lineages made it necessary to take more than one graft from the same donor and to give some hosts two transplants, from different donors. To increase the number of grafts that could be obtained from a fish, the caudal fin of the donor was often split into two halves along the midlateral plane and the spleen (in large donors), divided into two parts. In grafting, the transplant is inserted into a slitlike pocket cut into the musculature of the caudal peduncle. The suspensorium of the fin graft is pushed into the pocket with a blunt needle, while the external portion of the fin protrudes from the mouth of the pocket. As a consequence of being denervated, the transplanted fin initially undergoes degeneration, starting at its distal end, but six to eight days later, upon reinnervation, regeneration ensues. Spleen and heart grafts are pushed into similar pockets. Grafts are prevented from falling out by muscle contraction around the pocket. Nevertheless, two days after the operation all hosts were examined under a dissecting microscope to learn whether any graft had been lost for mechanical reasons.

Attempts to transplant scales according to the method of Hildemann (1957) proved impractical for two reasons. The scales of *Rivulus* are small and delicate and the integument is covered with a heavy layer of mucus that makes it difficult to insert a scale graft into a scale pocket of the host. In these fish, it is also difficult to distinguish a successfully transplanted scale from the many scales of the host that are similar in size and color.

The age of laboratory-reared donors ranged from 20 to 163 days post-hatching and that of the hosts from 34 to 536 days. In addition, one donor (NSU) and two hosts (FT, NA) were brought in from the wild already fully mature sexually and were perhaps about a year old. All fish were maintained in isolation from other fish, in 40% sea water (distilled water and filtered sea water) at temperatures ranging from 21° C. to 29° C. and averaging 25° C.; they were fed on brine shrimp nauplii and mosquito larvae (*Aedes* sp.). Their solid wastes and uneaten food were siphoned out each day; the water was filtered once a week, and changed completely at the first signs of cloudiness. With the exception of a single fish that died within three months after the operation, all hosts were maintained for seven months or longer.

RESULTS

Intra-sib grafts were made in 26 different host-donor combinations (Table I) to determine whether sibs possess identical genotypes. Five hosts (#8, 10, 14, 16, 18) each received two grafts from the same donor to bring the total number of intra-sib grafts to 31. In these five combinations, the hosts were of appreciably larger size than the donors, the dorsal fin of the donor being much smaller than the scales of the host, and it was feared that because of their small size the grafts might become damaged or might be resorbed, even when host and donor possessed compatible genotypes. Two grafts were therefore implanted into the hosts in the hope that at least one of the grafts would fulfil the surgical requirements for survival.

Table I

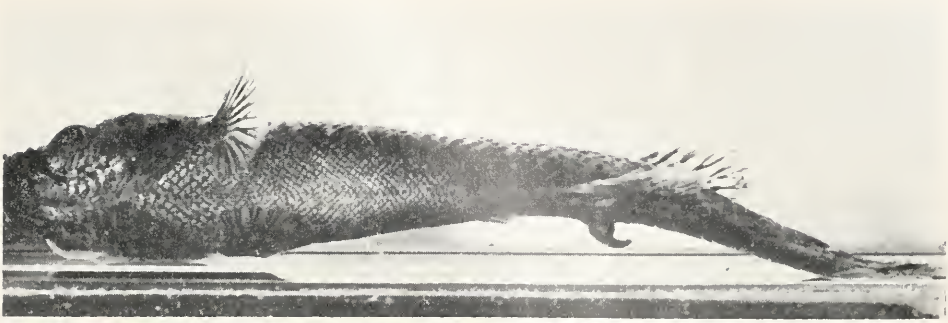
Fate of intra-sib grafts in Rivulus marmoratus

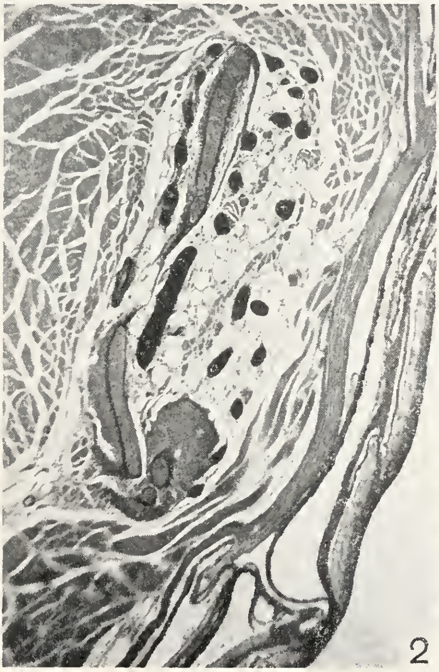
	Donor	Host	Age of host	Type of graft	Fate of graft	Criteria of graft survival	Time (days)
1	*FT*-m	*FT*-e	34–39*	spleen	+	normal histology	363**
2	-n	-c	34–49	caudal fin	+	fin distorted, projects from body wall at site of implantation.	364
3	-o	-b	34–39	caudal fin	+	as in #2	730***
4	-p	-g	34–39	caudal fin	+	as in #2	374
5	-q	-h	34–39	spleen	+	as in #1	363
6	-s	-i	34–39	caudal fin	+	as in #2	364
7	-t	-j	34–39	caudal fin	+	as in #2	366
8	-u	-k	163–168*	heart, spleen	++	heart beating, both grafts possess normal histology	236
9	-v	-k	163–168	anal fin	−	no trace of graft found in serial sections	236
10	-v	-l	163–168	heart, spleen	++	as in #8	235
11	-u	-l	163–168	anal fin	+	fin imbedded in musculature, normal histology	235
12	-w	-d	163–168	dorsal fin	+	as in #11	237
13	*NA*-a	*NA*-b	87	anal fin	+	as in #11	216
14	*DS*-M	*DS*-c	258	heart, spleen	+−	heart beating, no trace of spleen found in serial section	237
15	-M	-D	252	anal fin	+	as in #11	237
16	-α	-H	143	heart, anal fin	++	heart beating, fin as in #11	484
17	-α	-P	93	caudal fin	+	as in #11	237
18	*DS*-AB	*DS*-AE	263	heart, caudal fin	++	as in #16	236
19	*DS*-AC$_{\gamma}$	*DS*-ACJ	120	heart	+	heart beating, normal histology	236
20	-ACZ	-ACJ	120	caudal fin	+	as in #11	236
21	-AC$_{\gamma}$	-ACH	122	spleen	+	as in #1	235
22	-ACZ	-ACH	122	anal fin	+	as in #11	235
23	-ACZ	-ACE	127	spleen	+	spleen bright red at graft site	240
24	-AC$_{\gamma}$	-ACE	127	anal fin	+	as in #2	240
25	-ACZ	-ACA	159	heart	+	as in #19	237
26	-AC$_{\gamma}$	-ACA	159	caudal fin	+	as in #2	237

* Age of host at time of operation not known exactly, since parent was not monitored for eggs during a five-day period.

** Number of days after the operation at which hosts died or were sacrificed and condition of grafts ascertained.

*** Host still alive and fin graft in excellent condition 730 days after the operation.





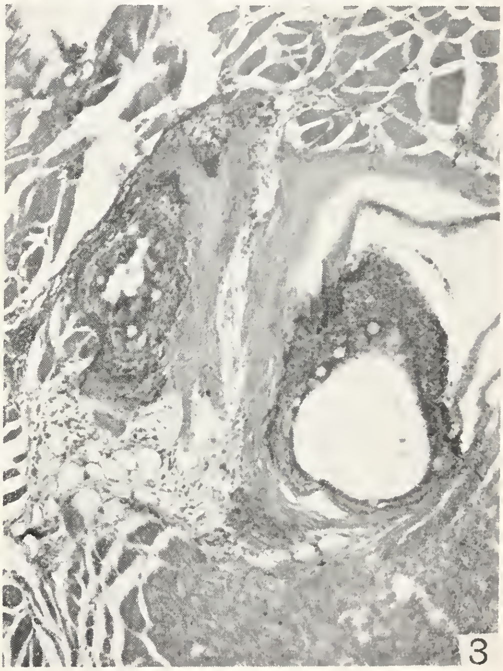


FIGURE 1. Ventral view of *Rivulus* (*NL-A*) bearing a caudal fin transplant from one of its offspring (*NL-AA*) on its right side slightly above anal fin. Transplanted fin is deformed. Photographed 514 days after the operation. × 2.5.

FIGURE 2. Cross-section through the proximal portion of a caudal fin transplant (*NA-a* → *NA*). The two large cellular bony elements are hypural bones of the basal plate of the caudal fin. The dark acellular bones (circular) are the external fin rays. Notice the loose connective and adipose tissue between the fin rays and the periosteal layer surrounding the hypurals. Host was sacrificed 234 days after the operation. × 180.

FIGURE 3. Cross-section through the distal end of an anal fin transplant (*DS-α* → *DS-H*) which had been deeply inserted into the musculature of the caudal peduncle. No external fin rays regenerated, but inside the skeletal muscle the fin epidermis had rounded up and formed a vesicle. Numerous goblet cells, very typical for *Rivulus*, can be seen in the epidermal layer facing the vesicle. The dermis borders at the musculature. In the proximal region of the graft (not shown here) the other elements of typical fins were found. Host was sacrificed 484 days after the operation. × 180.

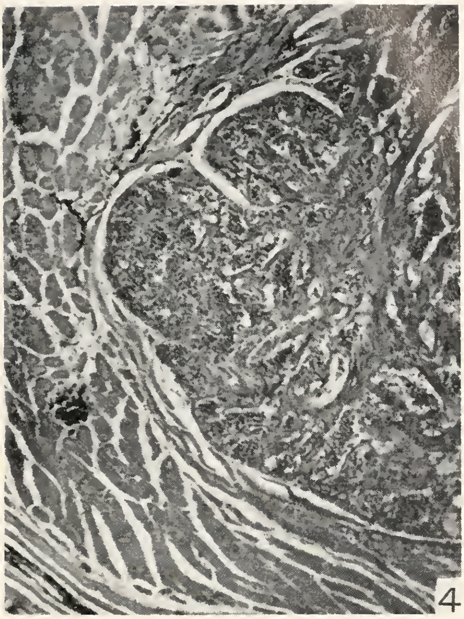
The fate of seven fin transplants could readily be ascertained by macroscopic examination at the time when the hosts were sacrificed. The transplants had grown into typical fins which, however, were distorted as a result of the twisting and injury of the fin rays at the time of the operation (Fig. 1). The fate of nine other fin grafts was verified by histological examination. In these hosts, the fin grafts, which were very small, had been pushed deep into the musculature of the host, and its integument had closed over the mouth of the pocket before the fin could regenerate. Serial sections revealed all the elements of typical fins imbedded in the musculature (Fig. 2). In some cases the fin epithelium of the "external fin" had formed a vesicle (Fig. 3), the inside of which was lined by the fin epidermis with its characteristic mucus cells. Failure to regenerate an external fin in these cases has nothing to do with an immunological reaction. It merely resulted from the fact that the fin had been inserted rather deeply into the pocket.

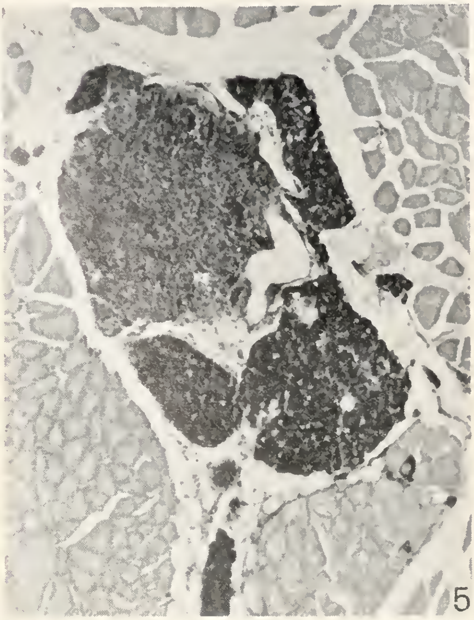
The heart grafts became vascularized within three to four days after the operation and some of them resumed their rhythmic contraction as early as the second day after the operation. All heart transplants were still beating at the time the hosts were sacrificed. Histological examination of all heart transplants failed to reveal any degenerative changes (Fig. 4).

The spleen graft could often be seen through the skin as a dark red structure imbedded in the musculature. The transplanted spleens proved indistinguishable histologically from the host spleens (Fig. 5). It should be noted that two of the hosts, *FT-c* and *FT-d*, had also received a second transplant from a donor belonging to a different line (Table III). These inter-line grafts were rejected. At the end of the experiment all but two of the 31 intra-sib grafts were found. One of these two, host *FT-k*, had received a spleen and a heart graft from *FT-u*, both pushed deeply into the musculature of the caudal peduncle. At the same time, the anal fin of a second donor, *FT-v*, was implanted just anteriorly to the heart and spleen grafts. During the operation the anal fin was damaged. On the seventh postoperative day the fin and the area around the pocket were greatly inflamed and the graft appeared to be disintegrating. Fourteen days after the operation only a few fin rays were seen. There was no indication of any regeneration. The host was finally sacrificed 236 days after the operation. The heart and spleen grafts were present and intact, but no trace of the anal fin could be found. The authors are of the opinion that the failure of this graft to survive is more likely the result of injury during the operation than of immunological reaction. The second exceptional host, *DS-c* (#14), received a heart and spleen graft from *DS-M*, implanted in close proximity. The spleen graft could be observed through the skin for five weeks, but when the fish was sectioned 237 days after the operation, a perfectly normal heart was found but no trace of the spleen. Again the authors share the opinion that this graft failure was not caused by an immunological reaction, but possibly in this case by resorption owing to the extremely small size of the graft.

In Table II are listed six "offspring to the parent" graft combinations. The survival of grafts in this combination is the chief criterion for the occurrence of uniparental reproduction (Kallman, 1962). All six fin grafts survived. Similarly, the two "F_2 into nonparental F_1" combinations were also successful.

Because of a lack of suitable fish, we could only test two "parent to offspring" combinations. This combination can be used to determine whether the parent is


4


5

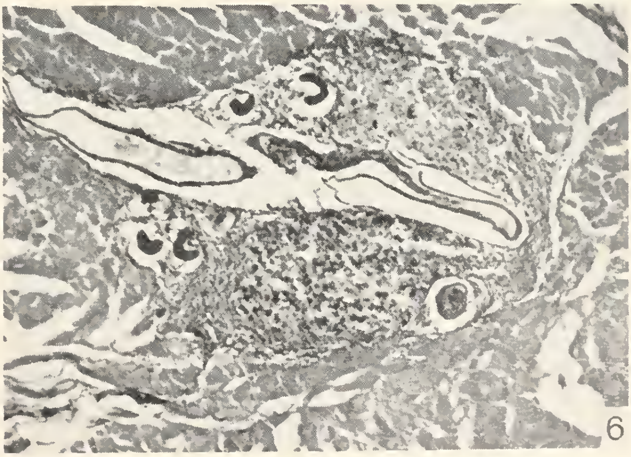

6

FIGURE 4. Cross-section through part of a heart transplant (*DS-α* → *DS-H*). Host sacrified 484 days after the operation. × 180.

FIGURE 5. Cross-section through a transplanted spleen to which inadvertently some pancreatic tissue adhered (*NSU* → *NSU-H*). Host sacrified 234 days after the operation. × 180.

FIGURE 6. Cross-section through the site of a disintegrated caudal fin graft (*NL-AB* → *NSU-i*). The soft tissue around the fin rays has been completely destroyed and replaced by host connective tissue and histiocytes. Notice the remnants of the fin rays (dark-staining crescent-shaped structures) and the remnants of two disintegrated hypural bones. Host sacrificed 290 days after the operation. × 180.

still heterozygous with different alleles segregating during gametogenesis. If this is so, then certain alleles of the parent may not be represented in the offspring and certain tissue antigens of the parent may be "foreign" to the offspring. The offspring, therefore, may reject parental tissue grafts. In our two combinations, both grafts survived.

In order to determine whether any of the six wild-caught *Rivulus* were members of the same clone, we performed eight interline grafts in seven out of 16 possible host donor combinations (Table III). Of these, a caudal fin graft from an *NSU* survived in a *DS* host, indicating that *NSU* and *DS* and their descendants belong to the same clone. All other transplants disintegrated. The caudal fin from the *NL* donor implanted in an *NSU* host exhibited the chronic type of graft rejection. When the host was fixed 290 days after the operation and histologically examined, remnants of the disintegrated graft were still observed (Fig. 6). The chronic rejection of tissue grafts is usually found in cases where host and donor differ by only a few histocompatibility loci. Since in uniparentally reproducing species new clones arise by the accumulation of mutations, the existence of clones differing from each other by few genes is to be expected.

TABLE II

Fate of intraline grafts in Rivulus marmoratus *between hosts and donors belonging to different generations*

#	Donor	Host	Age of host	Type of graft	Fate of graft	Criteria of graft survival	Time (days)
				Offspring to Parent Grafts			
27	*FT*-X	*FT*		caudal fin	+	fin imbedded in musculature, normal histology	355*
28	-W	*FT*		anal fin	+	as in #27	234
29	*NA*-a	*NA*		caudal fin	+	as in #27	234
30	*NL*-AA	*NL*-A	407	caudal fin	+	fin projects from body wall	613
31	-BA	-B	407	caudal fin	+	as in #27	220
32	*NSB*-AA	*NSB*-A	266	caudal fin	+	as in #27	234
				Parent to Offspring Grafts			
33	*NSU*	*NSU*-B	73	spleen	+	normal histology	235
34	*NSU*	*NSU*-H	507	spleen	+	normal histology	234
				F_2 into Nonparental F_1			
35	*NL*-AB	*NL*-B	535	anal fin	+	fin distorted, projects from body wall at site of implantation, normal histology	92
36	*NSB*-AA	*NSB*-C	266	caudal fin	+	as in #35	241

* Number of days after the operation at which hosts died or were sacrificed and condition of graft ascertained.

DISCUSSION

These results are in agreement with the conclusion that these fish are self-fertilizing hermaphrodites (Harrington, 1961, 1963). Since self-fertilization represents the ultimate in inbreeding, these fish must have achieved a high degree of homozygosity in which identical alleles segregate during gametogenesis and the offspring possess a genotype identical with that of the parent.

Billingham and Silvers (1959) have pointed out that the most sensitive indicator for homozygosity is the tissue transplantation test; Kallman (1962) has discussed its application to the study of uniparental reproduction in vertebrates. The test is predicated on the fact that in vertebrates tissue transplants from one individual to another of the same species only succeed if all, or almost all, of the donor's antigens are also present in the host. The presence or absence of tissue antigens is under the control of specific genes, called histocompatibility genes (for reviews see Snell, 1957; Medawar, 1959; Owen, 1959). The test is valid only if a large number of genes are concerned with transplantation immunity, because otherwise two individuals selected at random might be identical with respect to their histocompatibility genes, yet heterozygous and thus different with respect to many other loci. For this test to be valid, the histocompatibility genes must also exist at least in two or more allelic states and be scattered randomly over the chromosomes. If these conditions are fulfilled, the chances that two individuals selected at random from a large interbreeding population would have compatible genotypes are extremely small. In the mouse the number of histocompatibility genes has been estimated to be at least 13–16 (Barnes and Krohn, 1957; Prehn and Main, 1958), and a similar number has been reported for the rat (Billingham *et al.*, 1962). In the teleost, *Xiphophorus maculatus*, at least twelve histocompatibility genes have been demonstrated (Kallman, unpublished data).

TABLE III

Fate of interline grafts in Rivulus marmoratus

#	Donor	Host	Age of host	Type of graft	Fate of graft	Criteria of graft survival	Time (days)
I	*NSU*-C	*DS*-AC	289	caudal fin	+	fin imbedded in musculature, normal histology	237*
II	*FT*-a	*DS*-A	506	heart, spleen	– –	no trace of grafts found in serial sections	240
III	*NA*-a	*DS*-ACF	125	spleen	–	as in #II	234
IV	*NSU*-F	*FT*-b	163	caudal fin	–	as in #II	376
V	*NL*-AB	*FT*-c	163	heart	–	as in #II	236
VI		died					
VII	*NL*-BA	*FT*-d	163	anal fin	–	scar tissue present	365
VIII	*NL*-AB	*NSU*-i	504	caudal fin	–	scar tissue present	290
IX	*NL*-AB	*NSB*-B	266	dorsal fin	–	as in #II	377

* See Table II.

The low estimates of four to six histocompatibility genes for the guinea pig (Bauer, 1960) and only three for the Syrian hamster (Billingham *et al.*, 1960) should be considered in the context of the unitary origin of the strains employed. All laboratory strains of the Syrian hamster can be traced back to two females and a single male belonging to a litter of twelve captured in 1930. The hamster strains that have come into existence during the last 33 years probably have been created in the same way as many other laboratory strains. They have been founded by one, two or three breeding pairs obtained from colonies existing elsewhere. That this founder principle has led to a general decay of the genetic variability, accompanied by the fixation of certain alleles and loss of others through genetic drift, is most likely. Similar results indicating a small number of histocompatibility genes have recently been reported by Billingham and Silvers (1963) for another subspecies of the hamster, *M. a. brandti.* Without additional experiments, however, it is still premature to conclude that hamsters, in contrast to mice, rats and platyfish, have only a few transplantation antigens. Wild-caught individuals are usually considered heterozygous for a large number of loci, but such an assumption may not always be justified and it has yet to be shown just how heterozygous hamsters are in nature. If the reports are true that the hamster is rather uncommon, then the effective breeding population within a particular area will be relatively small, resulting in a certain degree of homozygosity. In this case, even the survival of some skin transplants exchanged among sibs of wild-caught parents could be understood. A strikingly similar situation has recently been described for two disjunct populations of the platyfish, *Xiphophorus couchianus* (Kallman, 1964). A high percentage of transplants exchanged among the offspring of wild-caught females were permanently accepted, provided the females had been collected in springs where the population density of this species was lowest. Similar results have been obtained with certain wild-caught *X. variatus* and *X. maculatus* (Kallman, unpublished).

No valid estimate of the number of histocompatibility genes has been presented for any other species, but since it is almost the universal experience of biologists that homotransplants exchanged among wild-caught animals of the same species or among members of heterozygous strains are rejected, it is likely that large numbers of histocompatibility genes are present in most species. Tissue transplantation experiments have been performed in twelve species of teleosts (for references see Kallman, 1964), all wild-caught or heterozygous stocks, but not a single transplant survived with the exception of those in *X. couchianus*, *X. variatus* and *X. maculatus*.

The transplantation test, therefore, is valid and the survival of almost all intra-line grafts in *Rivulus* can be taken as excellent evidence that the descendants of each wild-caught fish are identical genetically and constitute a clone. No segregation of histocompatibility genes had taken place and therefore the fish were presumably homozygous not only for these genes but for most of the genome. The last point, of course, cannot be settled with absolute finality, because of the logical, though biologically remote, possibility of an unknown mechanism that keeps these fish permanently heterozygous through successive generations. Conceivably such a mechanism would entail the nonviability of all homozygotes, but this possibility seems highly unlikely, since under optimum laboratory conditions, at least, with intensive monitoring of eggs, the number of eggs failing to develop was low. There is also the possibility that the eggs may develop gynogenetically after being activated by the fish's own sperm. This question will have to be settled, eventually, through cytological analysis.

The prevalence of normal development in *Rivulus* is no serious obstacle to the view that these fish are homozygous. Homozygosity would have been gradually achieved with enough time for the elimination of lethal and deleterious genes, in contrast to meiotic parthenogenesis in which homozygosity may be achieved in a single step. Salekhova (1963) reported that in *Serranus scriba,* artificially self-fertilized eggs develop normally, but better survival was obtained in cross breeding.

Kallman (1962, 1963) showed that natural populations of *M. formosa* consist of a number of clones, the frequencies of which remained rather constant from year to year. It is very likely that a similar condition will be found in *Rivulus.* Although the results of our clonal analysis are preliminary because of the small numbers of fish, they show that at least two fish derived from parents collected in different places evidently belong to the same clone. Of these parents, *DS* and *NSU* (*cf.* Table III for their descendants used as host and donor), one was collected June 13 and the other June 24, 1960, the first to the north and the second to the south of a long-established road running due east to the open lagoon and thus forming a barrier between north and south expanses of the marsh. Each fish would be trapped in the depression of the march where it was caught, except when high tides and heavy rains flood the marsh, which is only during the autumn months. The effective distance between the two fish then would involve movement east to the lagoon shore, north or south along the lagoon to pass the road barrier, and west in the other marsh expanse, 420 + 405 + 375 yards, or *vice versa.* In sum, a minimum route of 1300 yards, open in its entirety only one season of the year, would have to be traversed by a fish travelling between these two collecting sites.

It is interesting to determine whether other vertebrates that normally reproduce uniparentally are also homozygous. In the gynogenetic cyprinid, *Carassius auratus gibelio,* the egg nucleus is in the metaphase of the second meiotic division when the eggs are deposited and fertilization takes place (Lieder, 1959). Forty minutes later the second polar body is extruded. The female pronucleus possesses the haploid chromosome number, but before the first cleavage division is completed, the diploid condition has been reestablished. Lieder (1959) could not determine how this is accomplished, but states that there is no fusion of the egg nucleus with the second polar body. The diploid condition may therefore be reestablished by chromosome doubling without cell division and the fish, consequently, would be homozygous. It is not possible to determine with the transplantation test whether the gynogenetic teleost, *M. formosa,* is homozygous or heterozygous (Kallman, 1962). Regardless of whether the fish arises from haploid or diploid eggs, parent and offspring possess identical genotypes and a "three-way histocompatibility" among parent and offspring exists.

On the basis of a few cytological preparations it appears that individuals of the parthenogenetic subspecies of the lizard, *Lacerta saxicola,* are also homozygous. Darewski and Kulikowa (1961) state that the first meiotic division is normal and that the diploid condition is reestablished either as a result of the fusion of the products of the second meiotic division or the suppression of the first cleavage division. Both processes result in homozygosity.

Several sporadic cases of uniparental reproduction in vertebrates have to be considered "abnormal" and these offspring all appear to be heterozygous. No transplantation experiments were performed with Spurway's guppies, which are produced by self-fertilization, but a segregation of pigment genes among the uniparental offspring clearly indicated that they were heterozygous as expected in a first, "selfed" generation.

The heterozygous nature of male parthenogenetic turkeys was proven by transplantation tests. Healey *et al.* (1962) showed that there existed only a "one-way histocompatibility" between parthegenones and parent; grafts from the offspring survived in their respective parents, but all parental grafts were rejected by the offspring, as were grafts between parthenogenetic sibs. Chromosome segregation, therefore, had taken place and parent and offspring did not possess identical genotypes. The question whether the parthegenones were homozygous or heterozygous was settled by an additional transplantation experiment (Poole *et al.*, 1963). Offspring sired by the parthegenones rejected grafts from their male parent. This clearly indicated that the parthegenones were heterozygous.

In *Gambusia affinis* a similar "one-way histocompatibility" between parthenogenetic offspring and parent was observed by Kallman and Kilby (unpublished). Only grafts from offspring to parent survived. Whether these fish were homozygous or heterozygous could not be determined since the parthegenones failed to reproduce.

SUMMARY

1. Since self-fertilization constitutes the ultimate basis of inbreeding, a species or race of self-fertilizing hermaphrodites should consist of clones, all members of which possess identical genotypes and are homozygous. This prediction has been tested on a recently discovered population of *Rivulus marmoratus* from the east coast of Florida. All wild-caught specimens of this population tested so far have proved to be hermaphrodites. Those kept in isolation in aquaria, and their hermaphrodite descendants kept in isolation *ab ovo*, have reproduced by self-fertilization.

2. The transplantation test has been used to determine whether fish that had descended from the same wild-caught progenitor possess identical genotypes. Fins, spleens and hearts were transplanted in 36 different host-donor combinations involving six different lines (sib to sib, parent to offspring, offspring to parent). Only two transplants failed to survive, but their loss may have been due to mechanical reasons. These results are in accordance with the theoretical prediction that these fish are largely homozygous.

3. Transplants were performed in seven different inter-line combinations. Of these only a single graft survived, indicating that the fish collected on different days in different places belonged to the same clone.

LITERATURE CITED

BARNES, A. D., AND P. L. KROHN, 1957. The estimation of the number of histocompatibility genes controlling the successful transplantation of normal skin in mice. *Proc. Roy. Soc. London, Ser. B*, **146**, 505–526.

BAUER, J. A., JR., 1960. Genetics of skin transplantation and an estimate of the number of histocompatibility genes in inbred guinea pigs. *Ann. N. Y. Acad. Sci.*, **87**: 78–92.

BILLINGHAM, R. E., AND W. K. SILVERS, 1959. Inbred animals and tissue transplantation immunity. *Transpl. Bull.*, **6**: 399–405.

Billingham, R. E., and W. K. Silvers, 1963. The hamster. *Sci. Amer.*, **208**: 118–127.

Billingham, R. E., B. A. Hodge and W. K. Silvers, 1962. An estimate of the number of histocompatibility loci in the rat. *Proc. Nat. Acad. Sci.*, **48**: 138–147.

Billingham, R. E., G. H. Sawchuck and W. K. Silvers, 1960. Studies on the histocompatibility genes of the Syrian hamster. *Proc. Nat. Acad. Sci.*, **46**: 1079–1090.

Clark, E., 1959. Functional hermaphroditism and self-fertilization in a serranid fish. *Science*, **129**: 215–216.

Comfort, A., 1961. The longevity and mortality of a fish (*Lebistes reticulatus* Peters) in captivity. *Gerontologia*, **5**: 209–222.

Darewski, I. S., and W. N. Kulikowa, 1961. Natürliche Parthenogenese in der polymorphen Gruppe der kaukasichen Felseidechse (*Lacerta saxicola* Eversmann). *Zool. Jahrb., Syst.*, **89**: 119–176.

Harrington, R. W., Jr., 1961. Oviparous hermaphroditic fish with internal self-fertilization. *Science*, **134**: 1749–1750.

Harrington, R. W., Jr., 1963. Twenty-four-hour rhythms of internal self-fertilization and of oviposition by hermaphrodites of *Rivulus marmoratus*. *Physiol. Zoöl.*, **36**: 325–341.

Harrington, R. W., Jr., and L. R. Rivas, 1958. The discovery in Florida of the cyprinodont fish, *Rivulus marmoratus*, with a rediscription and ecological notes. *Copeia*, **1958**: 125–130.

Healey, W. V., P. S. Russel, H. K. Poole and M. W. Olsen, 1962. A skin grafting analysis of fowl parthenogens: Evidence for a new type of genetic histocompatibility. *Ann. N. Y. Acad. Sci.*, **99**: 698–705.

Hildemann, W. H., 1957. Scale homotransplantation in the goldfish (*Carassius auratus*). *Ann. N. Y. Acad. Sci.*, **64**: 775–791.

Hubbs, C. L., and L. C. Hubbs, 1932. Apparent parthenogenesis in nature, in a form of fish of hybrid origin. *Science*, **76**: 628–630.

Hubbs, C. L., and L. C. Hubbs, 1946. Breeding experiments with the invariably female, strictly matroclinous fish, *Mollienisia formosa*. *Genetics*, **31**: 218.

Kallman, K. D., 1960. Dosage and additive effects of histocompatibility genes in the teleost *Xiphophorus maculatus*. *Ann. N. Y. Acad. Sci.*, **87**: 10–43.

Kallman, K. D., 1962. Gynogenesis in the teleost, *Mollienesia formosa* (Girard), with a discussion of the detection of parthenogenesis in vertebrates by tissue transplantation. *J. Genet.*, **58**: 7–21.

Kallman, K. D., 1963. Population structure of the all-female gynogenetic teleost, *Mollienesia formosa* (Girard). *Proc. XVI Int. Con. Zool.*, **2**: 170.

Kallman, K. D., 1964. Genetics of tissue transplantation in isolated platyfish populations. *Copeia*, in press.

Kallman, K. D., and M. Gordon, 1958. Genetics of fin transplantation in xiphophorin fishes. *Ann. N. Y. Acad. Sci.*, **73**: 599–610.

Lieder, U., 1955. Männchenmangel und natürliche Parthenogenese bei der Silberkarausche *Carassius auratus gibelio* (*Vertebrata, Pisces*). *Naturwiss.*, **42**: 590.

Lieder, U., 1959. Über die Eientwicklung bei männchenlosen Stämmen der Silberkarausche *Carassius auratus gibelio* (Bloch) (Vertebrata, Pisces). *Biol. Zentralbl.*, **78**: 284–291.

Maslin, T. P., 1962. All-female species of the lizard genus *Cnemidophorus*, Teiidae. *Science*, **135**: 213.

Medawar, P. B., 1959. Zoologic laws of transplantation. *In:* Transplantation of Tissues (ed. by L. A. Peer), The Williams and Wilkins Co., Baltimore.

Olsen, M. W., 1962. The occurrence and possible significance of parthenogenesis in eggs of mated turkeys. *J. Genet.*, **58**: 1–6.

Owen, R. D., 1959. Genetic aspect of tissue transplantation and tolerance. *J. Med. Educ.*, **34**: 366–383.

Poole, H. K., W. V. Healey, P. S. Russel and M. W. Olsen, 1963. Evidence of heterozygosity in parthenogenetic turkeys from homograft responses. *Proc. Soc. Exp. Med. Biol.*, **113**: 503–505.

Prehn, R. T., and J. M. Main, 1958. Number of mouse histocompatibility genes involved in skin grafting from strain Balb/cAn to strain DBA/2. *J. Nat. Cancer Inst.*, **20**: 207–209.

Reinboth, R., 1962. Morphologische und funktionelle Zweigeschlechtlichkeit bei marinen Teleostiern (Serranidae, Sparidae, Centracanthidae, Labridae). *Zool. Jahrb. Physiol.,* **69**: 405–480.

Salekhova, L. P., 1963. On self-fertilization and development of self-fertilized eggs of *Serranus scriba* (L.) *Voprosy ichtyologii,* **3**: 275–287.

Snell, G. D., 1957. The homograft reaction. *Ann. Rev. Microbiol.,* **11**: 439–458.

Spurway, H., 1957. Hermaphroditism with self-fertilization and the monthly extrusion of unfertilized eggs in the viviparous fish *Lebistes reticulatus. Nature,* **180**: 1248–1251.

Suomalainen, E., 1962. Significance of parthenogenesis in the evolution of insects. *Ann. Rev. Entomol.,* **7**: 349–366.

White, M. J. D., 1954. Animal Cytology and Evolution, 2nd ed. Cambridge University Press, Cambridge, England.

REVERSIBLE INHIBITION OF SWIMMING IN STOMOTOCA ATRA BY MESOGLEAL EXTRACTS OF SOME OTHER MEDUSAE

HOWARD M. LENHOFF[1]

Laboratory for Quantitative Biology, University of Miami, Coral Gables 46, Florida, and Friday Harbor Laboratory, University of Washington, Friday Harbor, Washington

When large numbers of the two predacious jellyfishes, *Stomotoca atra* and *Aequorea aequorea,* are collected in the same container of sea water, specimens of the smaller of the two hydromedusae, *S. atra,* sink to the bottom and remain there indefinitely. If, however, these non-swimming *Stomotoca* medusae are then isolated and placed in fresh sea water, they rise to the surface within 5 minutes and swim with their normal vigor. The unusual reaction of *Stomotoca atra* to the presence of *Aequorea* appears to result from a "tetanic" contraction of its velum. The causative agent is a heat-stable, dialyzable, non-toxic substance present in *Aequorea* and certain other medusae.

MATERIALS AND METHODS

The organisms were collected at Friday Harbor, Washington, in the summer of 1962. The medusae were removed from the sea in a glass vessel, rather than in a net, in order to preserve the integrity of their delicate tissues. The animals were ordinarily used within four hours after capture. Fresh sea water was employed in most of the experiments. The temperature was kept at 13° C. by setting the experimental glass containers on a sea table of running sea water.

RESULTS

1. Diffusion of an inhibitory substance from intact Aequorea aequorea and from isolated pieces of its mesoglea

In a typical experiment, a specimen of *A. aequorea* (60 mm. in diameter and 30 ml. in volume) was placed in a dish of 200 ml. sea water. The *Aequorea* was pulsating, the manubrium was closed, and the surface epithelium was not visibly broken. A single *Stomotoca* was then placed in the dish of sea water containing the *Aequorea.* The *Stomotoca* swam actively for about 6 minutes before any lessening of swimming could be noted. After 30 minutes, however, it was barely moving. By 45 minutes, it ceased swimming and assumed a contracted state.

Next, a piece of *Aequorea* mesoglea, free of epithelium and approximately 15 ml. in volume, was placed into 100 ml. of sea water containing two *Stomotoca* medusae. The swimming movements of the medusae were inhibited at 45 and 60 seconds, respectively; the animals were still inhibited 8 hours later.

These experiments show that (a) intact *Aequorea* medusae emit a substance which inhibits the swimming activities of *Stomotoca atra,* (b) the inhibitory substance is present in a freely diffusible form in the mesoglea, and (c) this substance can slowly (but not readily) pass through an epithelial barrier.

---

[1] Investigator of the Howard Hughes Medical Institute.

2. *Inhibition studies using mesogleal fluids*

Aequorea mesogleal fluids, which were presumed to contain a high concentration of the inhibitory agent, were acquired by scraping off the dorsal epithelium, carving out and removing a conical piece of mesoglea, and allowing the mesogleal fluids to exude into the apex of the cone-shaped well.

Specimens of *Stomotoca* were inhibited from swimming within 10 to 15 seconds after being placed in a 1/10 dilution of this mesogleal fluid. Proportionally slower inhibitions, by 20 to 50 seconds, took place in all animals kept in a 1/20 dilution. At 1/40, some animals were inhibited at 30 seconds and others as late as 11.6 minutes. In addition, some of these inhibited animals partially recovered their swimming movements in the subsequent 4.3 minutes. At 1/100 dilutions, inhibitions started at 7.5 minutes. The latter animals usually recovered within 40 minutes while remaining in the same solution.

3. *Preparation of large amounts of mesogleal fluid*

Large portions of the mesoglea were excised free of epithelia and twisted in a clean cotton cloth until most of the fluid was expressed. Since *A. aequorea* is about 96% water (Hyman, 1940), the volume of fluid collected was nearly equal to the volume of mesoglea placed into the cloth. The expressed fluid, diluted with sea water, inhibited the swimming of *Stomotoca* at dilutions almost identical with those of the mesogleal fluid obtained from the cone-shaped cavity in Expt. 2.

It can be assumed that the concentration of the inhibitor in specimens of *Aequorea* of the same size is probably similar from animal to animal since dilutions of these expressed fluids consistently inhibited the swimming of *S. atra* within 20 to 50 seconds. (The time required for inhibition to occur varied somewhat with the size of *Stomotoca*, the smaller medusae being inhibited more quickly than the larger ones.) Thus, specific dilutions of the mesogleal fluids can represent reliable and quantitative expression of the relative amounts of the inhibitory agent. Since 1/20 dilutions consistently induced unequivocal and rapid inhibitions, this dilution was used as the testing concentration in all subsequent experiments.

4. *Hydrogen ion concentration of the mesogleal fluid*

The pH of the undiluted *Aequorea* mesogleal fluid was 7.6; that of the 1/20 dilution was 7.91, which is identical to the pH of sea water. Therefore, we can exclude a difference in hydrogen ion concentration as the cause of the inhibition.

5. *Effects of heat and dialysis on the inhibitory properties of the mesogleal extract*

Twenty-five ml. of the mesogleal fluid were heated for 15 minutes to 85° C. and then diluted 1:20. This diluted, heat-treated solution, like the untreated fluid, inhibited the swimming of *Stomotoca* in less than 30 seconds.

A 20-ml. aliquot of a fresh extract was dialyzed against 80 ml. of sea water. After 8 hours a portion of the water surrounding the bag was diluted to a volume which would give a theoretical concentration of 1/20 of the original fluid, assuming complete dialysis. This dilute solution inhibited two specimens of *Stomotoca* within 30 and 50 seconds, respectively. An equivalent of a 1/10 dilution inhibited the swimming of the medusae within 5 seconds.

The above experiments show that the active component of the mesogleal fluid is heat-stable and dialyzable—and, therefore, probably a small molecule. Accordingly, the inhibitory action is not likely due to any immunologically active macromolecule.

6. *Origin of inhibitory substance*

Aqueous extracts of the epithelia of *Aequorea* were prepared and tested for inhibitory activity as follows: First the bulk of the mesoglea of *Aequorea* was removed and extracted. Next, the remaining parts of the animal, which contained nearly all the cells and some mesoglea, were expressed through a cotton cloth. At 1/20 the mesoglea extract took 20 to 30 seconds to act, while the "cell" extract inhibited in less than 5 seconds. At 1/100 the mesoglea extract inhibited at 40 and 150 seconds; after 10 minutes, the same solution—with the same animals—was further diluted to 1/200 and the animals recovered by 6.1 minutes. In contrast, the extracts of *Aequorea* cells at 1/100 dilutions inhibited at 5 and 20 seconds; these inhibited animals did not recover by the subsequent 1:2 dilution. Furthermore, at 1/200 the mesogleal fluids had no visible inhibitory action, while the cell extracts inhibited at 25 and 40 seconds.

These experiments indicate that the inhibiting substance originates in the epithelial cellular areas of *Aequorea*, and that some then diffuses into the mesoglea and out of the animal. This observation suggests hitherto unexplored transport and storage functions for the mesoglea.

It is possible that part of the greater activity of the cell extract might be due to some nematocyst toxin released while the extract was prepared; this seems unlikely, however, because the inhibitor is found in the mesoglea which is free of epithelia and nematocysts.

7. *Reversibility of the inhibition*

Specimens of non-swimming *Stomotoca* found in the collection bucket with *Aequorea* medusae resumed swimming within minutes after being placed in fresh sea water, indicating that the inhibition was reversible. To test for reversibility, medusae that were inhibited by a 1/20 dilution of mesogleal extract for 10 minutes were placed in 600 ml. of fresh sea water. Within 75 seconds the medusae went from the contracted non-swimming state to a relaxed state. By 95 seconds, they pulsated occasionally. At 150 seconds the pulsations were forceful enough to cause slight swimming movements, and by 165 seconds the animals were swimming actively.

These general orders of events occurred with all animals, regardless of the length of time they were previously exposed to the inhibitor. Also, the shorter the exposure to the inhibitor, the more quickly active swimming movements were resumed in fresh sea water. For example, animals exposed for 5 minutes recovered in two minutes, while those inhibited for 20 minutes recovered within 5–8 minutes. All medusae kept in the 1/20 diluted extracts for 12 hours swam actively within 11 minutes when placed in fresh sea water.

Furthermore, animals kept in the 1/20 diluted *Aequorea* extracts for 24 hours, although inhibited for most of that period, gradually overcame the inhibition and recovered their swimming movements without being placed in fresh sea water.

In addition, by establishing the reversibility of the inhibitions, these experiments indicate that under the conditions used, the inhibitor was not toxic.

8. Action of Aequorea mesogleal extracts on intact and dissected parts of Stomotoca atra

When a *Stomotoca* is placed in the diluted mesogleal extract (1/20) the mouth bends from side to side. Next, the velum and radial canal contract, the velum converting from a circular to a "square" shape with "corners" equidistant between the radial canals. This latter contraction is probably responsible for the cessation of the swimming movements.

The mesogleal extracts (1/20) had similar effects on freshly dissected parts of *Stomotoca.* The isolated mouth (with attached gonads) twisted from side to side. Isolated tentacles contracted, coiling and uncoiling like a spring. In a "quarter" of the umbrella, the single radial canal and the accompanying velum contracted. Finally, a ring of the isolated velum (5 cm. long) coiled immediately and remained in the coiled state as long as it was in the extracts. When this coil of velum was placed into fresh sea water, it elongated somewhat and began a series of regular contractions.

9. Specificity of response

Neither *Sarsia tubulosa, Sarsia flammea, Phialidium hemisphericum, Halistaura, Gonionemus, Probosidactyla,* nor the ctenophore, *Pleurobracia* (a prey of *Aequorea*), were inhibited by a 1/20 dilution of the *Aequorea* mesogleal fluids. The *Probosidactyla* medusae, however, did show some signs of "discontentment" after 30 minutes. Thus, of the animals tested, the inhibitory response was specific to *Stomotoca atra.*

10. Specificity of inhibitor source

Extracts of other medusae were tested for inhibitory activity. Since it was difficult to remove large amounts of clean mesoglea from these other forms, I used extracts of the whole animal. Of the medusae tested, only extracts of *Halistaura* were effective at 1/200 dilution (as was *A. aequorea*). *Sarsia tubulosa* extracts showed activity at 1/100 dilution, although the *Stomotoca* started to recover within a few minutes after the inhibition. Extracts of *Phialidium hemisphericum* were active both at 1/20 and 1/100 dilutions, although within an hour animals from the 1/200 experiment swam occasionally, while some from the 1/100 experiment were swimming vigorously. Only extracts of *Stomotoca* itself had no inhibitory action. When placed in a 1/20 dilution of the whole *Stomotoca,* the test animals swam as vigorously as the controls throughout the 12-hour observational period.

DISCUSSION

The small, two-tentacled, predacious jellyfish, *Stomotoca atra*—when exposed to extracts from other medusae—is reduced to a contracted state during which swimming ceases. This induced inhibition is markedly different from the relaxed state of *Stomotoca* observed during the transient cessation of swimming that follows its normal pulsating movements.

Not enough is known about the behavior of these organisms in nature to attribute any functions to this response as yet. At first I erroneously conjectured that since *Stomotoca atra* is a predator of other jellyfishes, the prey evolved a substance which essentially "narcotized" the predator. This is not plausible because even *Phialidium,* a common food of *Stomotoca,* contains this substance. Furthermore, it seems unlikely that in the ocean sufficient inhibitor would accumulate in the general area of these rapidly pulsating medusae to have any physiological importance.

Alternatively, the inhibitor might be considered a common metabolite of most medusae. Since the mouth of *Stomotoca* would be in close contact with the prey (the source of the inhibitor), then the mouth would be the first (and perhaps only) part of the *Stomotoca* to contract. And these contractions might aid *Stomotoca* in feeding by stimulating a suction-type peristalsis. The mouth was active in capturing and ingesting live prey even after I removed from *Stomotoca* its two tentacles or complete umbrella. Thus, this proposed chemical induction of a suction-type of feeding may be a useful adaptation by *Stomotoca* enabling it to ingest prey medusae without employing the manipulating actions of its tentacles.

This report shows that *Stomotoca atra* exhibits an unusual and specific response to extracts of other medusae. The nature of the inhibitory substance(s), the site(s) of its action, and the mechanism of its action invite further investigation.

I wish to thank Dr. Dixy Lee Ray, Dr. Demorest Davenport, and Dr. Paulo Sawaya for their encouragement of this research.

SUMMARY

1. Specimens of *Stomotoca atra* ceased to swim when they were placed in the same container with *Aequorea aequorea* medusae.

2. This inhibition of swimming was also produced by pieces of the isolated mesoglea from *A. aequorea,* and by mesogleal extracts from the latter.

3. Methods for preparing large amounts of a "standard" mesogleal extract of *Aequorea* are described.

4. The inhibitory action of the fluids was not a result of change in pH.

5. The inhibitory agent from *Aequorea* mesogleal fluids was heat-stable and dialyzable.

6. Larger concentrations of the inhibitory substances were found in the non-mesogleal portions of *Aequorea.*

7. The inhibitions were completely reversible, even after 12 hours' constant exposure to a 1/20 mesogleal extract.

8. The mesogleal extracts affected individual parts of the dissected *S. atra.*

9. The swimming of no other medusae tested was affected by the mesogleal extracts of *Aequorea.*

10. Extracts from all medusae tested, except from *Stomotoca atra* itself, inhibited the swimming of the *S. atra.*

LITERATURE CITED

HYMAN, L. H., 1940. Observations and experiments on the physiology of medusae. *Biol. Bull.,* **79**: 282–296.

CARBON DIOXIDE TENSION AND SEXUAL DIFFERENTIATION IN HYDRA

HELEN D. PARK, CHRISTYNA E. MECCA[1] AND ANNE B. ORTMEYER

Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland

In the past few years an extensive literature on a "sex gas" (Loomis, 1959b) of *Hydra* has accumulated. Loomis (1954) reported results appearing (p. 146) "to indicate that the primary stimulus that induces sexual differentiation in *Hydra littoralis* is a critical lowering of the oxygen concentration of the medium." Loomis and Lenhoff (1956) next reported, however, that (p. 562) ". . . although reduced oxygen tension is generally found in sexual cultures, it is not the causative factor *per se*." The authors further stated (p. 562): "Attempts to maintain hydra in the asexual condition were found to demand (a) frequent changes of water, (b) uncrowded conditions or (c) extreme shallowness (2–3 mm)." These observations were in contrast to those of Park (1956) on mass cultures of a clone of *H. littoralis* maintained from 1950 to 1956, and also to the observations of Burnett (1961) on a mass culture of *H. oligactis*. Moreover, Park *et al.* (1961), using a clone of Loomis's stock of *H. littoralis*, found that uncrowded conditions were not sufficient to maintain the hydras in the asexual state.

Loomis (1957; see also 1959a, 1959b, 1959c, 1960 and 1961) next concluded that (1957, p. 738) ". . . sexual differentiation may be reversibly induced in *Hydra* by measures that control the pCO_2 of their aqueous environment." More recently and interestingly, however, Loomis (1959d, 1961) reported that sexual differentiation of *Hydra* could not be induced by measures that increase the pCO_2 of the aqueous environment unless some feedback was permitted between *Hydra* and this environment.

The lack of agreement between the reports cited above and those of Burnett (1961), Park (1956) and Park *et al.* (1961) make it important to investigate further the efficacy of increased environmental pCO_2 in inducing sexual differentiation of *Hydra*. It is of particular importance to test the effect of a number of different methods of controlling pCO_2, including Loomis's method (Loomis, 1957), on the Loomis stock of *Hydra*, as well as to test the effect of the Loomis method on other species of *Hydra*. The results of such tests are the subject of this report.

MATERIALS AND METHODS

Three species of *Hydra* from different sources were used: (1) *H. littoralis*, (2) *H.* (sp.), and (3) *H. pseudoligactis*.

H. littoralis individuals were descended from a single mature male of Loomis's stock. Mass cultures of this clone have been maintained in our laboratory for four years. A brief description of the course of differentiation and subsequent disappearance of male gonads is given here to clarify some of the criteria used. The first indication of differentiation of gonadal tissue is a thickening of the ectoderm, usually in the subhypostomal region, though sometimes more proximally. The next day one sees, proximal to the site of the original thickening, discrete, mammiform protuberances of the ectoderm. These are the mature gonads, which during successive days move progressively nearer the budding region (just distal to the junction of body and stalk). The more proximal they are, the smaller and less mammiform they are. Those nearest the budding region often have a ragged appearance, and in the budding region appear as barely visible snags of tissue. Before the complete disappearance of the gonads in the budding region, the ectoderm of the subhypostomal region may again thicken and the whole process of differentiation, maturation, and disappearance of gonads may recur. Under oil immersion we have seen motile sperm in squashed preparations of mammiform gonads.

---

[1] Present address, National Institute of Dental Research, National Institutes of Health, Bethesda 14, Maryland.

Individuals of the second species used were descended from a male purchased from the Carolina Biological Supply Company and believed to be *H. littoralis*. After completion of the experiments, however, the conclusion was reached (Dr. Helen Forrest, personal communication) that this is not *H. littoralis*, but a species as yet unreported in North America. The periodic sexual differentiation in mass laboratory cultures was similar to that found for the Loomis stock of *H. littoralis* (Park, 1961). This *Hydra* had been maintained in mass culture for 6 months prior to the first experiment.

The *H. pseudoligactis* individuals were descended from a culture obtained from the Gladstone, Oregon, laboratories of the Carolina Biological Supply Company. Mass cultures had been maintained in the laboratory for two months prior to the experiment. No sexual forms have been found in laboratory cultures which have now been maintained for two years.

Mass cultures of each of the three species have been maintained according to the methods of Loomis and Lenhoff (1956), at laboratory temperature (23° ± 2° C.). Unless otherwise noted, all animals selected at the start of each experiment were asexual and without buds. Selection was made under a stereoscopic microscope at 10 × magnification.

In all experiments, cultures were offered an excess of brine shrimp larvae once daily for one hour, cleaned and re-covered with a tap water solution of 100 mg./l. $NaHCO_3$, 50 mg./l. disodium salt of ethylenediamine tetra-acetic acid, and 50 mg./l. $CaCl_2$ (Ca-BVT), unless otherwise noted. Two culture situations were used: (1) 10 hydras per 15-ml. beaker containing 15 ml. culture solution. Newly dropped buds were removed just before daily feeding; (2) 100 hydras per finger bowl (63 mm. inside diameter) containing 40 ml. culture solution. Culture size was maintained by random discard during the daily cleaning.

Three methods of controlling environmental pCO_2 were used: (1) injection (Loomis, 1957), (2) bubbling gas mixtures through cultures, and (3) flow of gas mixtures over cultures.

The injection method of increasing the pCO_2 of cultures consists of injecting cultures with solution that has previously been shaken in a syringe with gas mixtures of varying percentages of CO_2 and O_2. The source of water used for the solution was not stated by Loomis in 1957, but Loomis and Lenhoff (1956), described it as *tap* water, which we also used. Instead of following the procedure described by Loomis in 1957 (p. 736) for exposing solution to 100% O_2, "The culture solution was shaken three times before use with a large excess of 100 per cent oxygen . . .," we bubbled 100% O_2 vigorously through one liter of solution in a two-liter suction flask for 20 minutes. The flask was then closed tightly and the solution drawn off as needed.

In the bubbling method, a gas mixture from a pressure cylinder flowed through rubber tubing into a medium porosity, ceramic filter stick placed beneath the surface of the solution in the culture vessel. Carbon dioxide-free air for controls was obtained by pumping room air through 20% KOH.

In the flow method, a gas mixture flowed from a pressure cylinder or an air pump at 75 cc. per minute into the stem of an inverted funnel suspended over a culture dish. There was a ⅛-inch space between the funnel and the rim of the dish.

Experiments and Results

(1) *Injection*

(a) Loomis stock

The injection procedure described in detail by Loomis (1957) was used on 10-hydra cultures twice daily for 14 days with 5.6%, 5% and 1.7% CO_2 in O_2, and 100% O_2. The experiments were done on four occasions over 18 months, each time comparing a single concentration of CO_2 with 100% O_2 and twice also with controls exposed to room air. The results are shown in Figure 1.

Although the four experiments yielded a diversity of results, a few general statements may be made: (1) Hydras began to differentiate sexually within 24 hours, regardless of treatment. (2) The percentage of sexual forms increased for 2–4 days, then decreased in varying degrees. (3) The decreases ranged from small in the 5.6% CO_2-treated cultures of experiment I to large in the 100% O_2-treated cultures of experiment III. (4) While exceptions were observed (notably in experiments I and III), the percentages of sexual forms tended to rise and fall together within an experiment regardless of treatment.

(b) *H.* (sp.) and *H. pseudoligactis*

Injection was used *once* daily for 18 days on 10-hydra cultures of *H.* (sp.) and *H. pseudoligactis.* Five cultures of *H.* (sp.) and 4 of *H. pseudoligactis* were injected with calcium-free BVT that had been exposed to 5.6% CO_2 in O_2, 5 cultures of *H.* (sp.) were in the solution flushed with 100% O_2, and 5 cultures of *H.* (sp.) and 4 of *H. pseudoligactis* remained open to room air. No *H. pseudoligactis* individuals became sexual. No *H.* (sp.) individuals in cultures injected with CO_2-equilibrated solution became sexual; one in solution flushed with 100% O_2 was sexual from day 7 to day 13, and five exposed to room air were sexual from day 9 to day 13.

The experiment was repeated on *H.* (sp.) a year later. This time no sexual differentiation occurred under any of the treatments.

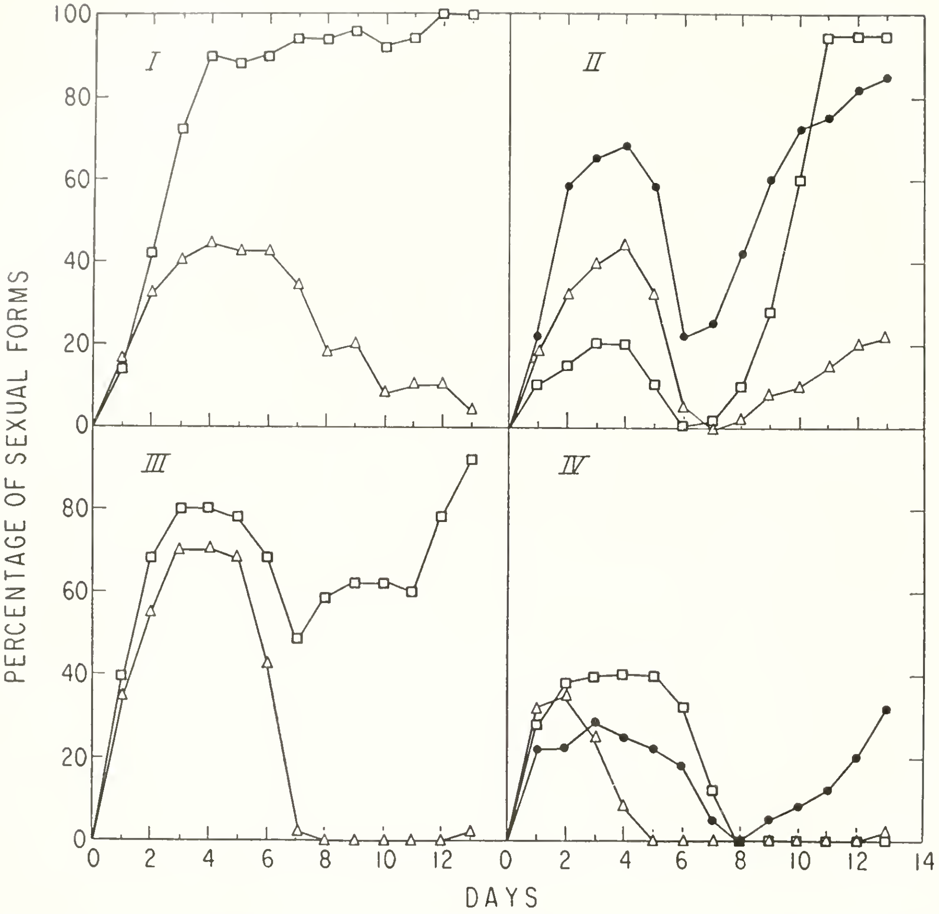


FIGURE 1. Four experiments showing percentage sexual forms in 10-hydra cultures exposed by the Loomis injection method twice daily to CO_2 (□) (5.6% in I and II, 5.0% in III, and 1.7% in IV), or to 100% O_2 (△), or to room air (●). Fifty hydras per curve in I, 40 in II, III and IV.

(2) *Bubbling (Loomis stock)*

(a) 5.0% CO_2 in air *vs.* CO_2-free air

One hundred-hydra cultures in calcium-free BVT were bubbled 22 hours daily for 40 days, one with 5% CO_2 in air, another with CO_2-free air. The second and third times this experiment was done a third culture was exposed to standing room air. The results are shown in Figure 2.

As in the injection experiments, hydras under *all* experimental conditions began to differentiate sexually within 24 hours. In cultures bubbled with CO_2-free air, as well as in those bubbled with 5% CO_2 in air, the percentage of sexual forms reached a maximum between days 4 and 6, then decreased rapidly. Cultures bubbled with 5% CO_2 in air showed a second increase in percentage of sexual forms, but even under this condition of elevated pCO_2, the percentage decreased again to zero in two replicates and remained below 15% in the third. A high, though variable, percentage of sexual forms in each of the undisturbed cultures in room air persisted from day 10 until the end of the experiment.

(b) Effect on sexual forms

Loomis (1959a) reported that (p. 266) ". . . hydra *de*differentiated to the asexual state a few days after turning on the aerator of the aquarium." To test the effect of bubbling on cultures containing both sexual and asexual hydras, the preceding experiment was slightly modified and was done three times. (1) Hydras were taken at random from the stock cultures so that on zero day, the experimental

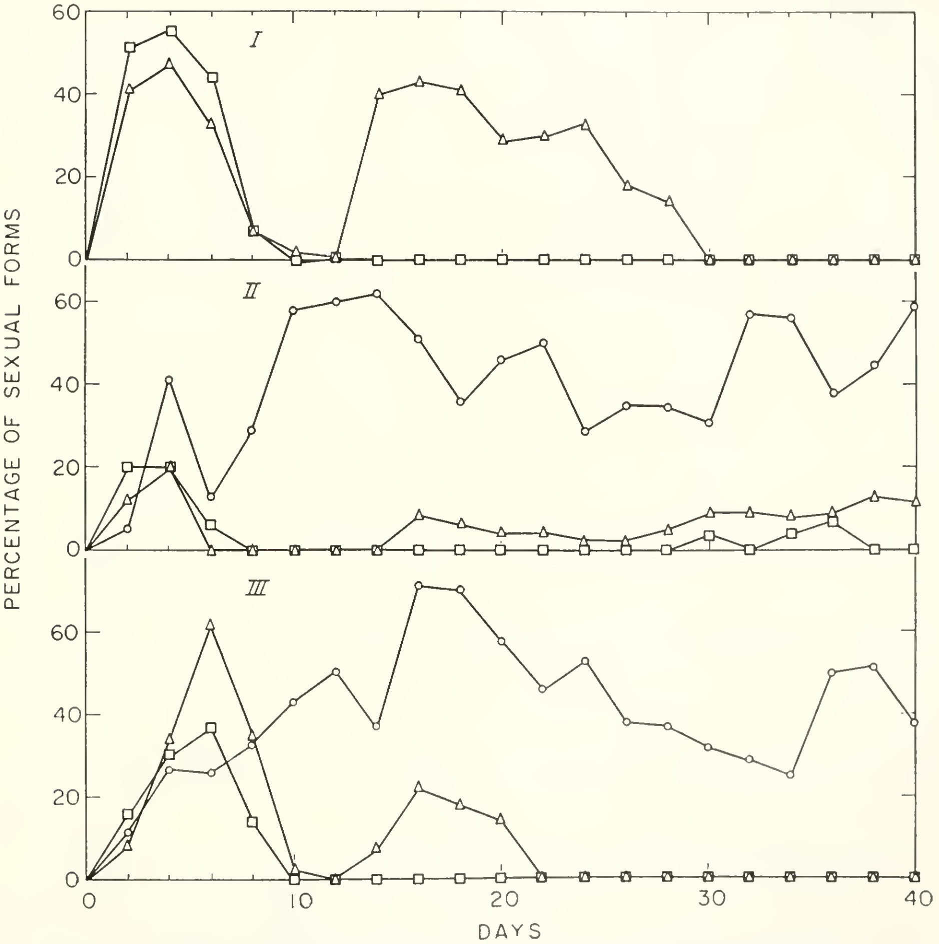


FIGURE 2. Three experiments showing percentage sexual forms in 100-hydra cultures bubbled 22 hours daily with 5% CO_2 in air (△), CO_2-free air (□), or exposed to room air (○).

cultures contained both sexual and asexual forms; (2) room air was used instead of CO_2-free air, and (3) the culture standing in room air was omitted. One replicate experiment was terminated on the fourteenth day. The results of the two 40-day replicates are shown in Figure 3.

In cultures bubbled with 5% CO_2 in air, as well as in those bubbled with room air, there was an immediate and rapid decrease in percentage of sexual forms. In the replicate experiment run 14 days, sexual forms in the 5% CO_2 bubbled culture decreased from 71% to 0 by day 10. In the culture bubbled with room air the decrease was 72% to 0 by day 12. It will be noted that one culture bubbled with 5% CO_2 in air remained asexual for the last 28 days of the experiment. In general, bubbling with 5% CO_2 was as effective as bubbling with room air in maintaining the hydras in an asexual condition.

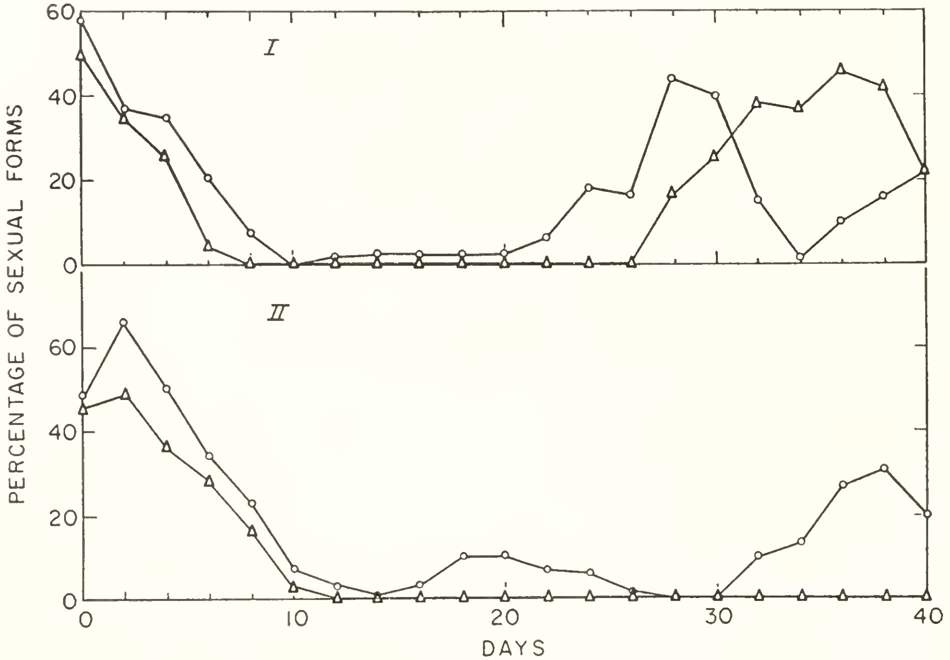


FIGURE 3. Two experiments showing percentage sexual forms in 100-hydra cultures bubbled 22 hours daily with 5% CO_2 in air (△), or room air (○). Both sexual and asexual forms on zero day.

(c) Bubbling two hours *vs.* a single injection daily

An experiment was done on 10-hydra cultures to compare bubbling two hours daily with injection once daily for 20 days. Four cultures in calcium-free BVT were bubbled with 5% CO_2 in O_2, four were injected with calcium-free BVT that had been equilibrated with 5.6% CO_2 in O_2, and four cultures remained undisturbed in room air between successive feeding and cleaning operations. When the experiment was repeated, four additional cultures were bubbled with 5% CO_2 in air. The results are shown in Figure 4.

Under all treatments hydras began to differentiate sexually within three days. In cultures bubbled with 5% CO_2 in O_2, the percentage of sexual forms reached a maximum by the fourth day, then decreased through the eighth day, and remained at less than 10 for the remaining 10 days of the experiments. In general, with the notable exception of a low percentage of sexual forms in the 5% CO_2-bubbled cultures throughout experiment I, there was considerable similarity in the results of the different treatments within each experiment.

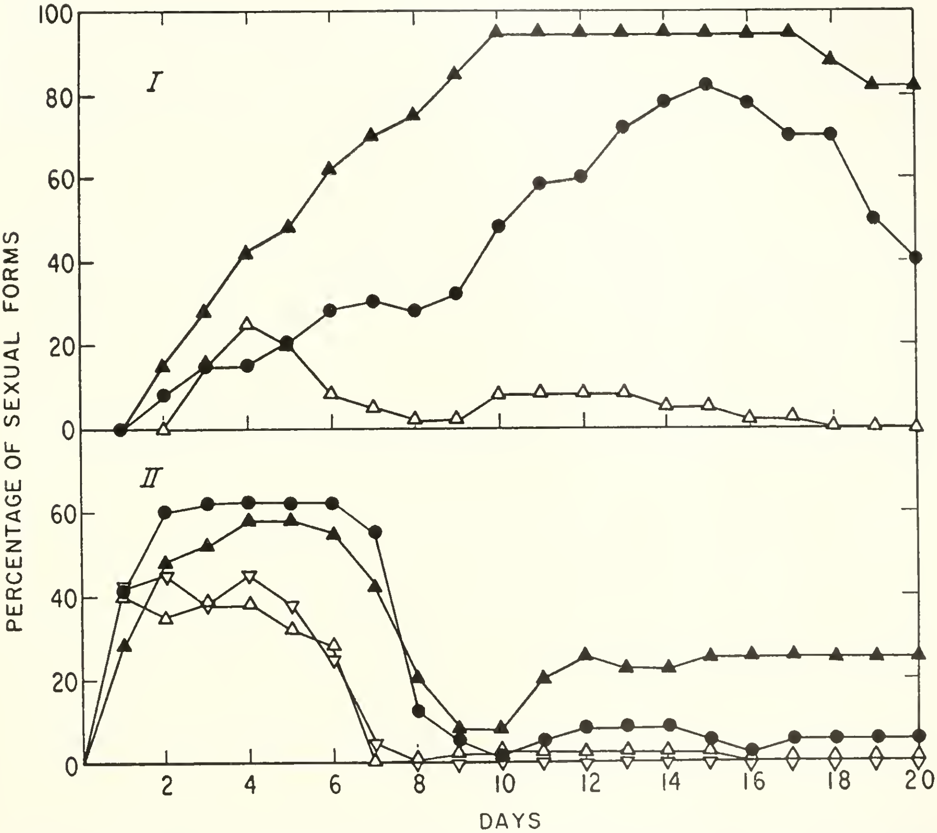


FIGURE 4. Two experiments showing percentage sexual forms in 10-hydra cultures injected once daily with 5.6% CO_2 in O_2-equilibrated solution (▲), or bubbled two hours daily with 5% CO_2 in O_2 (△), or 5% CO_2 in air (▽), or exposed to room air (●). Forty hydras per curve.

In experiment II, early sexual differentiation was quickly followed by disappearance of gonads under all treatments. By the tenth day there were almost no sexual forms in any of the cultures. As in the experiments with injection twice daily (see Figure 1), the percentages of sexual forms again tended to rise and fall

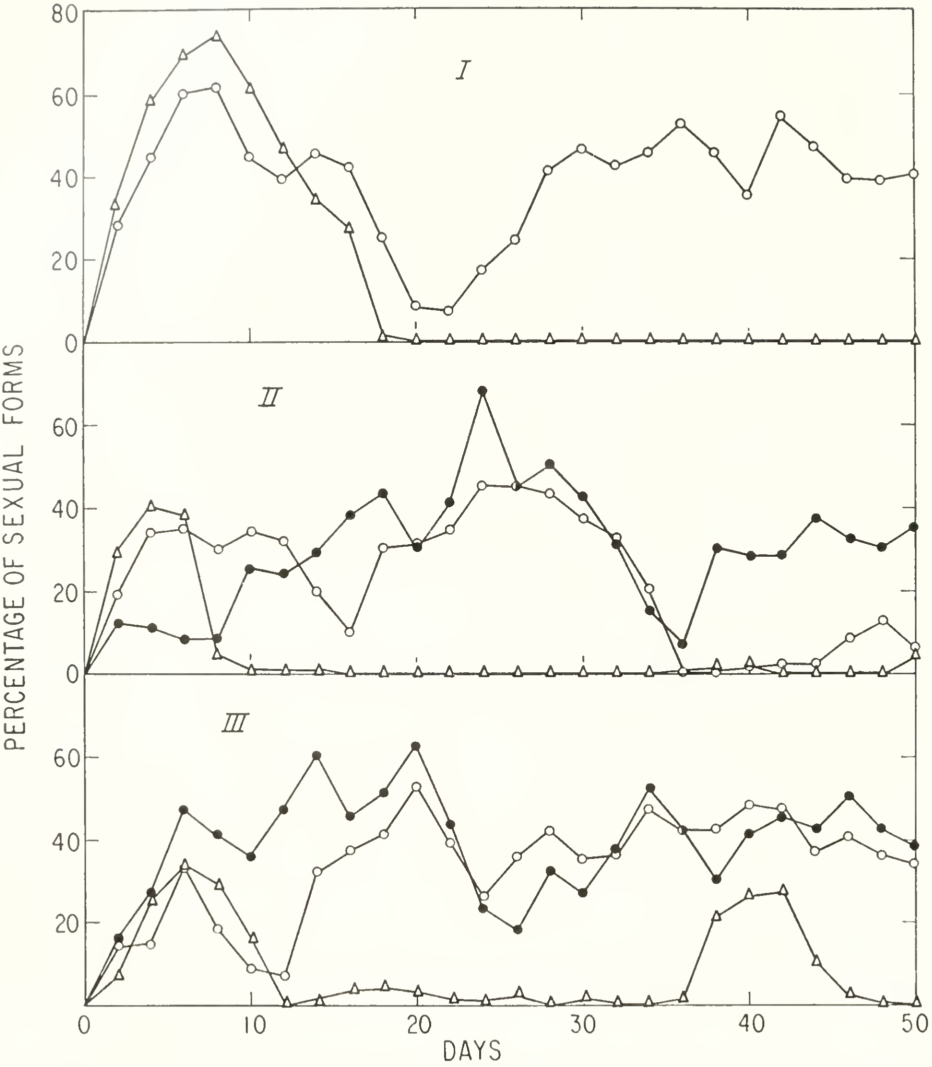


FIGURE 5. Three experiments showing percentage of sexual forms in 100-hydra cultures exposed to 5% CO_2 in air (△), or CO_2-free air (○), by flowing gas mixtures over the cultures. In two experiments a third culture was open to room air (●).

together, regardless of treatment. It is of interest that in the group of 4 cultures treated with CO_2 by injection, all of the sexual forms from day 13 to day 20 were in one culture while the other three cultures remained asexual.

(3) *Flow of gas mixtures* (*Loomis stock*)

One 100-hydra culture in calcium-free BVT, under a funnel, was exposed to 5% CO_2 in air and a comparable culture was exposed to CO_2-free air 22 hours daily for 50 days. The experiment was done three times. The last two times an additional 100-hydra culture was left open to room air. The results are shown in Figure 5.

Again, consistent with early sexual differentiation during other types of exposure, gonads began to develop within 24 hours in cultures under flowing gases, regardless of the mixtures used. Under CO_2 in air there was an increase in percentage of sexual forms for 4–8 days, followed by a rapid return to zero. There was almost no sexual differentiation for the following 24–40 days. By contrast, when cultures were exposed to CO_2-free air, large, though variable, percentages of sexual forms persisted throughout most of the experimental period. In each of the last two replicates, the percentages of sexual forms in cultures exposed to CO_2-free air were about the same for long periods as in cultures standing open to room air.

DISCUSSION

Asexual Loomis stock hydras developed gonads within a few days, regardless of whether the culture solution had a low pCO_2 (saturated with 100% O_2, CO_2-free air, or room air) or high pCO_2 (equilibrated with 1.7%, 5.0%, or 5.6% CO_2). We can, therefore, conclude that sexual differentiation in these hydras was not controlled by controlling the pCO_2 of the aqueous environment, within the limits of these experiments.

Loomis stock hydras were exposed to culture solution in which the pCO_2 was raised and maintained high by methods (continuous surface gassing and continuous bubbling) different from, and at least as efficient as, the injection method. In such experiments, essentially no new sexual differentiation occurred for as long as 30 days after the initial temporary gonad development. Elevated pCO_2 cannot, therefore, be said to promote or maintain sexuality under these conditions.

Furthermore, since no hydras of either the unidentified species or of *H. pseudoligactis* differentiated sexually when cultures were injected once a day with solution exposed to 5.6% CO_2, we can conclude that increasing the pCO_2 of the aqueous environment to this level does not induce sexual differentiation in these species.

Coupled with the results of our investigations, the progressive modifications by Loomis of the "sex gas" theory of sexual differentiation of *Hydra* present a number of aspects that merit discussion.

First, mention should be made of the wide variety of conditions in the laboratory under which the Loomis stock of *H. littoralis* differentiates sexually. In addition to sexual differentiation under environmental CO_2 tensions from zero to 5.6%, using three methods of controlling pCO_2, we have found that this stock may turn sexual in population densities ranging down to one hydra isolated in 10 ml. calcium-free BVT (Park *et al.*, 1961). Moreover, we have found that this stock differentiates sexually as readily and as often in a dilute salt solution (in double distilled water, the last distillation from glass) which contains neither bicarbonate nor ethylenediamine tetraacetic acid, as it does in calcium-free BVT (Park, 1961).

A second relevant point concerns time of sexual differentiation of *Hydra* in nature. Sexual forms of a number of North American species have been found in the *spring* of the year (Downing, 1909; Forrest, 1959, 1963; Griffin and Peters, 1939; Hyman, 1931; Trowbridge *et al.*, 1936; Whitney, 1907) from which we can conclude that the statement ". . . *Hydra* . . . turn sexual in the late fall . . ." (Loomis, 1961, p. 358) is an incautious generalization. Moreover, *H. littoralis*, the species on which the work leading to the "sex gas" theory was done, has been found sexual in March (Trowbridge *et al.*, 1936) as well as in the *fall* (Hyman, 1931). More recently, Forrest (personal communication) reported that she has collected sexual forms of *H. littoralis* in nature from late February to mid-October, the time of year varying with the locality.

A third point concerns the induction of sexual differentiation in certain clearly specified species of *Hydra* by lowering the temperature (see Brien, 1961, and Hyman, 1928 for reviews). It has been implied that stagnation had more to do with sexual differentiation of hydras in refrigerators than did lowered temperature (Loomis, 1959b). Nevertheless, the reports of experiments on *H. oligactis* (Brien and Reniers-Decoen, 1949); on *Pelmatohydra robusta* (Itô, 1954); on *H. parva* (Itô, 1955); and on *H. pirardi* (Brien, 1961; Burnett, 1961) clearly show that the refrigerated cultures that became sexual received the same care as the controls at higher temperatures and that the controls did not become sexual. We have confirmed the results on *H. oligactis* and *H. pirardi* and have also obtained similar results on *H. pseudoligactis*.

Finally, any theory attempting to explain the onset of sexual differentiation—or the onset of any other biological event—under laboratory conditions, ought, it seems to us, to take account of what is known about these biological events in nature. A case in point is the information available on sexuality in *H. littoralis* in its natural habitats. Three reports, Hyman (1931, 1938), Trowbridge *et al.* (1936) and Forrest (personal communication), state that sexual forms of this species were found in *swiftly* moving water. These observations show that sexual differentiation can occur where feedback between a hydra and its environment would be expected to play a minor role.

It seems safe to say that the inductor of sexual differentiation of *Hydra* either in nature or in the laboratory is still to be sought.

SUMMARY

1. One hundred- and 10-hydra cultures of *Hydra littoralis* and 10-hydra cultures of *H. pseudoligactis* and *H.* (sp.) were maintained 14–50 days in culture solutions equilibrated with gas mixtures varying in CO_2 content from 0.0% to 5.6%.

2. Two culture solutions and three methods of gassing cultures were used.

3. The results were:

   a. Asexual *H. littoralis* began to differentiate sexually within 1–4 days under all experimental conditions; initial sexual differentiation was temporary and was often followed by a second period of gonad development. In several experiments, percentages of sexual hydras tended to rise and fall together, regardless of treatment.

   b. *H. littoralis* cultures containing 46–72% sexual animals bubbled 22 hours daily with room air or 5% CO_2 in air were 95–100% asexual by day 12. Continued bubbling for the next 4 weeks with 5% CO_2 in air was as effective as bubbling with room air in maintaining asexuality.

c. No sexual differentiation occurred in *H. pseudoligactis* or *H.* (sp.) cultures exposed 18 days to elevated pCO_2. There was no sexual differentiation in *H. pseudoligactis* cultures exposed 18 days to room air. However, 5% of *H.* (sp.) in room air were sexual from day 9 to day 13.

LILTERATURE CITED

Brien, Paul, 1961. Étude d'*Hydra pirardi* (Nov. Spec.). Origine et répartition des nématocystes. Gamétogenèse. Involution postgamétique. Évolution réversible des cellules interstitielles. *Bull. Biol. France et Belgique,* **95**: 301–363.

Brien, Paul, and M. Reniers-Decoen, 1949. La croissance, la blastogénèse, l'ovogénèse chez *Hydra fusca* (Pallas). *Bull. Biol. France et Belgique,* **83**: 293–386.

Burnett, Allison L., 1961. The growth process in Hydra. *J. Exp. Zool.,* **146**: 21–84.

Downing, Elliot R., 1909. The ovogenesis of *Hydra. Zool. Zahrb.,* **28**: 295–324.

Forrest, Helen, 1959. Taxonomic studies on the hydras of North America. VII. Description of *Chlorohydra hadleyi,* new species, with a key to the North American species of hydras. *Amer. Midl. Naturalist,* **62**: 440–448.

Forrest, Helen, 1963. Taxonomic studies on the hydras of North America. VIII. Description of two new species, with new records and a key to the North American hydras. *Trans. Amer. Micr. Soc.,* **82**: 6–17.

Griffin, Lawrence E., and Dora C. Peters, 1939. A new species of *Hydra, Hydra oregona. Trans. Amer. Micr. Soc.,* **58**: 256–257.

Hyman, Libbie H., 1928. Miscellaneous observations on *Hydra,* with special reference to reproduction. *Biol. Bull.,* **54**: 65–97.

Hyman, Libbie H., 1931. Taxonomic studies on the hydras of North America. IV. Description of three new species with a key to the known species. *Trans. Amer. Micr. Soc.,* **50**: 302–315.

Hyman, Libbie H., 1938. Taxonomic studies on the hydras of North America. V. Description of *Hydra cauliculata,* n. sp., with notes on other species, especially *Hydra littoralis. Amer. Museum Novitates.* No. 1003. 9 pages. Pub. by Amer. Museum of Nat. Hist.

Itô, T., 1954. Studies on the reproduction of hydras. VI. Induction of the sexual reproduction in *Pelmatohydra robusta* (Itô) by lowering temperature. *Mem. Ehime Univ., Sect. II (Sci.) Ser. B. (Biol.),* **2**: 51–60.

Itô, T., 1955. Studies on the reproduction of hydras. VIII. Causative effect of lowering temperature on the sexual maturity in *Hydra parva* (Itô). *Bull. Marine Biol. Stat., Asamushi,* **7**: 120–126.

Loomis, W. F., 1954. Reversible induction of sexual differentiation in *Hydra. Science,* **120**: 145–146.

Loomis, W. F., 1957. Sexual differentiation in *Hydra.* Control by carbon dioxide tension. *Science,* **126**: 735–739.

Loomis, W. F., 1959a. Feedback control of growth and differentiation by carbon dioxide tension and related metabolic variables. *In:* D. Rudnick (ed.), Cell, Organism and Milieu, 17th Symposium, Soc. for the Study of Development and Growth (1958), The Ronald Press, New York, Chap. 9.

Loomis, W. F., 1959b. The sex gas of *Hydra. Sci. Amer.,* **200**: 145–156.

Loomis, W. F., 1959c. Control of sexual differentiation in *Hydra* by pCO_2. *Ann. N. Y. Acad. Sci.,* **77**: 73–86.

Loomis, W. F., 1959d. Further studies on cellular differentiation in *Hydra. Fed. Proc.,* **18**: 276.

Loomis, W. F., 1960. Cell differentiation: a problem in selective gene activation through self-produced micro-environmental differences of carbon dioxide tension. *In:* T. W. Goodwin and O. Lindberg (eds.), Biological Structure and Function, Proc. First IUB/IUBS Symposium (1960), Academic Press, New York, pp. 509–525.

Loomis, W. F., 1961. Feedback factors affecting sexual differentiation in *Hydra littoralis. In:* H. Lenhoff and W. Loomis (eds.), The Biology of *Hydra* and of Some Other Coelenterates; Univ. of Miami Press, Miami, Fla., pp. 337–362.

LOOMIS, W. F., AND H. M. LENHOFF, 1956. Growth and sexual differentiation of hydra in mass culture. *J. Exp. Zool.*, **132**: 555–572.

PARK, HELEN D., 1956. Some effects of x-radiation, magnesium sulfate and glutathione on *Hydra littoralis*. Ph.D. Thesis, The George Washington University.

PARK, HELEN D., 1961. Apparent rhythmicity in sexual differentiation of *Hydra littoralis*. *In:* H. Lenhoff and W. Loomis (eds.), The Biology of *Hydra* and of Some Other Coelenterates; University of Miami Press, Miami, Florida, pp. 363–372.

PARK, HELEN D., CHRISTYNA MECCA AND ANNE ORTMEYER, 1961. Sexual differentiation in *Hydra* in relation to population density. *Nature*, **191**: 92–93.

TROWBRIDGE, ALBERT H., ARTHUR N. BRAGG AND J. TEAGUE SELF, 1936. A new record of *Hydra littoralis* Hyman. *Okla. Acad. Sci. Proc.*, **16**: 28.

WHITNEY, DAVID DAY, 1907. The influence of external factors in causing the development of sexual organs in *Hydra viridis*. *Archiv. f. Entw.*, **24**: 524–537.

CARDIOREGULATION IN LIMULUS. I. PHYSIOLOGY OF INHIBITOR NERVES[1]

RALPH A. PAX[2] AND RICHARD C. SANBORN

Department of Biological Sciences, Purdue University, Lafayette, Indiana, and the Marine Biological Laboratory, Woods Hole, Mass.

Heart muscle contraction in the animal kingdom is initiated by two essentially different mechanisms (Prosser, 1961). In the first type, represented by vertebrate and molluscan hearts, muscle cells initiate the rhythmic contractions (myogenic hearts). In the second type, represented by most adult arthropod hearts, nerve cells initiate the rhythmic contractions, while the muscle cells possess no inherent rhythmicity (neurogenic hearts). Both neurogenic hearts and myogenic hearts are regulated by nervous connections with the central nervous system and, although the basis of the initiation of the contractions is different, a parallelism between the regulatory nerves in neurogenic hearts and those of myogenic hearts can be demonstrated. In both cases an inhibitory set of nerves which decreases and an excitatory set which increases the heart rate are present. Vertebrate (Mitchell, 1956) and molluscan (Welsh, 1953, 1957) cardioregulatory nerves have been extensively investigated, but less information is available on cardioregulatory parameters of arthropods. The most extensive studies have been performed on the hearts of decapod Crustacea (reviewed by Maynard, 1961; and by Florey, 1960).

In a brilliant series of experiments, A. J. Carlson (1905, 1909) characterized the heart of *Limulus polyphemus* as neurogenic. At the same time he demonstrated the existence of both excitatory and inhibitory cardioregulatory nerves. The excitatory nerves have their source in the abdominal ganglia and enter the cardiac ganglion at several points. The inhibitory nerves originate in the last two pairs of nerves leaving the hind-brain and travel in the seventh and eighth pairs of dorsal nerves until they reach the heart at about the fifth or sixth heart segments (Patten and Redenbaugh, 1900; Lochhead, 1950). The original studies of Carlson have not been followed by further detailed studies of the function of the regulatory nerves in *Limulus*. We report here some of the parameters of function of the inhibitory nerves of *Limulus*.

MATERIALS AND METHODS

Source and maintenance of animals

Animals were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. Some experiments were performed at the Marine Biological Laboratory, the majority were carried out at Purdue University. Animals remained healthy for more than six weeks in moist excelsior at ambient temperatures below 21° C.

---

[1] Aided by a grant (G-11234) from the National Science Foundation.

[2] Predoctoral fellow of the National Institutes of Health.

Recording

Recordings were made from "excised" preparations by removing the prosomal exoskeleton lateral to the heart on one side and dissecting the seventh and eighth cardiac nerves free of surrounding tissue (see Patten and Redenbaugh, 1900, and Lochhead, 1950, for anatomy and terminology). The anterior two or three segments of the heart were thus visualized. Electrical activity was recorded by means of platinum wire electrodes placed on the cardiac ganglion. Sea water or the saline solution of Chao (1933) (0.44 M NaCl, 0.009 M KCl, 0.037 M $CaCl_2$) were used to supplement the blood.

The method described above has drawbacks. (1) The stretch normally applied to the heart by the supporting ligaments is destroyed. (2) The normal flow of blood into the heart during diastole is destroyed. Both of these factors are known to influence the heart inotropically and chronotropically (Carlson, 1907). For these reasons a method of recording heart rate from the intact animals has been developed:

A 1 × 1 cm. piece of dorsal exoskeleton, directly over the heart in the region of the sixth heart segment, was removed. With care, removal without damage to the underlying hypodermis and without loss of blood is possible. Small-gauge insulated platinum electrodes were then inserted through the epidermal tissue to the cardiac muscle or ganglion. Placement of the electrodes was checked by the correlation between the electrical activity and the movement of the heart, as indicated by the simultaneous movement of the overlying hypodermal tissue. If visual monitoring was not needed, electrodes were simply passed through small holes drilled into the carapace overlying the heart.

Electrical activity was amplified with an A.C. preamplifier, displayed on an oscilloscope, and recorded simultaneously with a magnetic tape recorder.

Mechanical recording

In addition to measurement of the electrical activity, records of the mechanical movement of the heart are desirable. This is especially true when electrical stimulation is used since it causes interference with the electrical record. For such recordings a 3-0 stainless steel insect pin was bent into a hook, a small hole drilled in the same region used for the electrical recordings, and the pin inserted through the epidermal tissue and hooked into the dorso-lateral heart muscle. The pin was connected to a Statham force transducer. The small opening in the epidermal tissue, the low blood pressure of the animal and the quick clotting time of the blood prevent any great loss of blood while performing these operations.

Stimulation of inhibitory nerves.

Inhibitory nerves were stimulated with a square-wave stimulator *via* stainless steel insect pins or platinum wires. Such stimulation is complicated by the fact that it has been impossible completely to isolate the heart from the animal without disrupting connections of the inhibitory fibers to the cardiac ganglion. Previous workers have compromised and dissected the heart as free as possible from the animal, but we feel that it is desirable to record heart contractions in an animal which has been manipulated as little as possible. We, therefore, place most confidence in the studies employing "intact" preparations. For these, stimulation was accomplished with electrodes penetrating the ventral integument medial to the first branchiothoracic muscle and posterior to the last pair of walking legs. In this region the inhibitory fibers anastomose and pass dorsally toward the heart.

The stimulus was normally given for a period of 30 seconds, followed by a 100-second rest interval. The sequence in which the various frequencies were presented was randomized to eliminate cumulative effects.

RESULTS

Normal heart rates

To establish a control value for the rate of beat, we have placed hearts excised from *Limulus* in sea water or in saline solution for five hours or more and have monitored their rates of beats continuously during this period. From the direct recordings so obtained, cursory monitoring disclosed no marked changes in rate over this period. A section of such a record is shown as Figure 1.

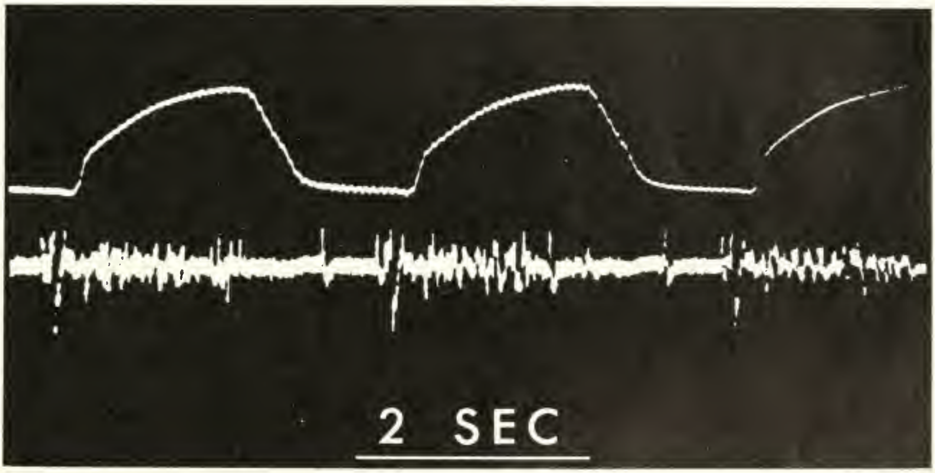


FIGURE 1. Normal electrical activity (lower record) and muscular contractions in an isolated heart (upper record). Upward deflection indicates contraction.

Sample intervals between ten contractions, chosen by use of a table of random numbers, from recordings of five animals over long periods were chosen, the instantaneous rates calculated and the mean frequency of beat and standard deviation were obtained by standard statistical techniques. The values so obtained were compared by a brief analysis of variance to determine the confidence which we could place in our further measurements.

Data for five hearts summarized in Table I show that while the mean rates so calculated range between 17.26 and 27.61 beats per minute, the variation in the rate for any one individual is small, as may be judged by the low standard deviation (1.89 compared with a mean value of 21.58 for all animals) of the rates.

The same analysis indicates that the variability between animals is so large (s = 12.28) that large numbers of animals would be required to make valid quantitative comparisons between animals. There is no correlation between heart rate and size, sex or season.

The heart beat of intact preparations can be monitored for several days. Direct observation for up to 24 hours showed no marked changes in rate of beat. Measurements similar to those carried out on the excised hearts disclosed mean rates ranging from 14.90 to 23.25 beats per minute. Once more, a comparison of the standard deviations shows that there is little point in comparing rates among individuals, but that the low standard deviation (s = 0.364) of the variability in rate of any one individual permits us to attach considerable significance to changes in rate greater than about one beat per minute.

Effect of inhibitory nerve activity

Stimulation of the inhibitory fibers of excised preparations resulted in a slowing of the rate. The magnitude of this slowing was dependent on stimulus strength and frequency. In a typical preparation, stimulus strengths of four volts or less (20 cycles/sec., 1-msec. duration) were ineffective while six volts or more caused a significant decrease in rate. In four other animals the minimum effective stimulation was between three and seven volts. Raising the stimulus strength above this threshold value resulted in no further increase in the effectiveness of stimulation. It therefore appears that all the inhibitory fibers are brought into action within a narrow range of stimulus strengths. Either there are comparatively few inhibitory fibers or they all have very nearly the same electrical characteristics.

TABLE I

Observations on 5 intact hearts and 5 excised hearts. Values are in beats per minute

	Intact	Excised
	17.17	22.18
	14.90	17.26
	23.25	21.49
	17.29	27.61
	20.90	19.35
$\bar{X}$	18.70	21.58
Standard Deviation (Intra-animal)	0.364	1.89
Standard Deviation (Inter-animal)	10.52	12.28

In the intact animal the relationship of change in heart rate to stimulus strength is essentially similar to that found in the excised preparations. Figure 2 is a typical record. In a typical case, a stimulus strength above 40 volts (20 cycles/sec., 1 msec.) was required before any significant slowing of the heart rate was noted. In five intact preparations, the threshold ranged from a low of about 20 volts to a maximum of slightly over 100 volts. The higher threshold in intact preparations is undoubtedly due to shunting of the stimulating current.

In a series of four intact preparations a study of the relationship between stimulus frequency and decrease in heart rate was made. Because of the variability among animals, discussed above, we have calculated the relative rate of contraction for each animal and compared these changes. We define the relative rate as the minimum rate of beat during stimulation, divided by the pre-stimulation rate for the same animal. Figure 3 shows the results of these experiments. The relationship between stimulus frequency and relative slowing of the heart is not linear. Maximum slowing of the heart is achieved by stimulus frequencies between 10 and 80 cycles/sec., although stimulation at frequencies as low as 2.5 cycles/sec. caused some slowing. At a frequency of stimulation of 200 cycles/sec. there was an increase in heart rate.

The response of the heart is not tightly coupled with the onset of stimulation of the inhibitory fibers. At the beginning of a period of stimulation the heart rate sometimes becomes gradually slower over a period of 10 to 15 seconds, *i.e.*, the time interval between beats becomes progressively greater for two or three beats before becoming stable at some new level. In a similar manner the end of stimulation and return of the rate to the normal level are not simultaneous. Half a minute is sometimes required before the rate has again returned to the prestimulation rate. This represents 5 or 6 beats in which the time interval between beats is becoming progressively less.

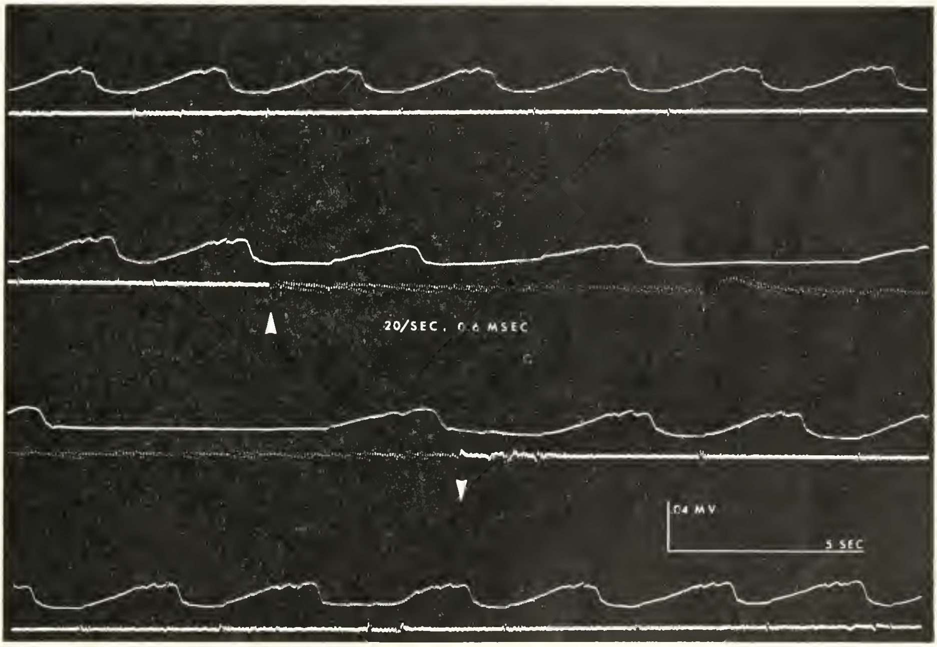


FIGURE 2. Changes in heart rate in an intact animal when inhibitory nerves are stimulated. The record is continuous from top to bottom. The upper record in each case is muscular contraction with increasing tension an upward deflection. The stimulus begins at the upward arrow and stops at the downward arrow.

If the inhibitory nerves are stimulated at some constant frequency for a period of 30 seconds, with intervening rest periods of 100 seconds, the maximum response is not constant from one stimulation period to the next. Instead, at times the heart may slow by as much as 60% but at other times slow by less than 1%. Figure 4 shows a typical case. This also indicates that there is no tight coupling between activity in the inhibitory nerves and heart rate. There was no apparent correlation between the relative decrease in rate with stimulation and the number of previous stimulations.

DISCUSSION

These experiments confirm the earlier observations of Carlson (1909) on the cardioinhibitory functions of extrinsic nerves in *Limulus*. They also extend our knowledge of some of the parameters of cardioinhibition in *Limulus*, since Carlson was unable to obtain quantitative results with available equipment.

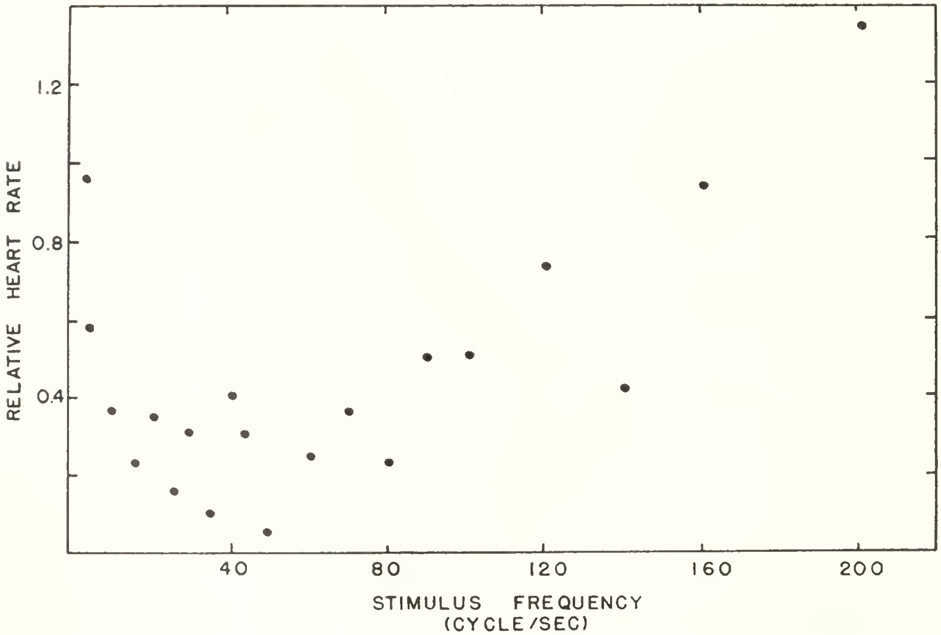


FIGURE 3. Relationship between heart rate and frequency of stimulation of inhibitory nerves in intact animals. Each point represents the average for four animals. Stimuli were at suprathreshold value and 1-msec. duration for 30 seconds. The average rate was calculated for the 30-second period.

The range of heart rates, from 14.9 to 27.5 per minute, which we observed are within the range of 8 to 28 previously reported for *Limulus* (Spector, 1956). There is little or no difference in the rate of beating of hearts in intact animals and the "excised" preparations. It therefore seems likely that the cardioregulatory nerves are probably not continuously biasing the heart rate but rather that they function only intermittently.

The range of heart rates which occur in *Limulus* makes quantitative comparisons of induced changes in rate between animals difficult. Since the rate for any particular animal varies only within narrow limits it is feasible to obtain quantitative results if we use one animal's heart rate as its own control. For example, in intact animals rate changes as small as one beat per minute can be assigned significance.

The graded responsiveness of the heart rate to stimulations of increasing frequencies is worth comparing with the results of similar experiments performed on the crayfish (Florey, 1960). In that crustacean the change in heart rate was also found to be a function of the frequency of stimulation of the inhibitory fibers. However, the response differed from that found in *Limulus*. In the crayfish, frequencies of stimulation less than 15 cycles/sec. are ineffective while frequencies greater than 35 cycles/sec. result in complete standstill of the heart. In the range of frequencies of stimulation between 15 and 35 cycles/sec. the decrease in heart rate rises almost exponentially as the frequency of stimulation is increased.

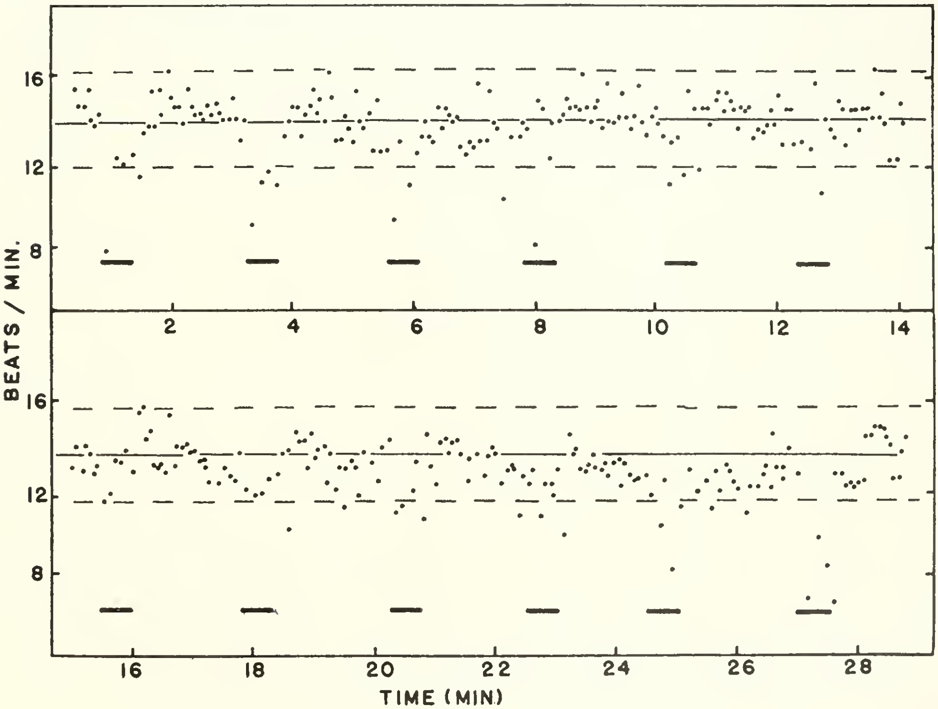


FIGURE 4. Variability in heart rate with constant stimuli. Stimuli were at 20 cycles/sec., 1-msec. duration for 30 seconds. Periods of stimulation are shown by the heavy horizontal lines at the bottom. The frequency of heart beat is in beats/min. Each point represents one beat. The solid horizontal line is the mean unstimulated rate and the dotted lines are three standard deviations on either side of the mean.

In *Limulus*, frequencies of stimulation of 2.5 cycles/sec. cause some slowing in the heart. As the frequency is increased, the heart rate does not continue to decrease but rather reaches a low point by 15 cycles/sec. and remains at this level until a frequency of stimulation of almost 90 cycles/sec. is reached. Frequencies above 90 cycles/sec. are less effective in slowing the heart than those below that level. At a frequency of stimulation of 200 cycles/sec. there is an actual increase in the heart rate. Carlson (1905) reported that with proper adjustment of the stimulus frequency and strength he was able to produce complete standstill of the *Limulus* heart. We were not able to duplicate these results.

From the differences in the response of the crayfish heart and the *Limulus* heart, it seems likely that in *Limulus*, but not in the crayfish, some excitatory fibers are contained in the inhibitory nerve. Since stimulation of the seventh and eighth nerves at high frequency causes a cardiac acceleration, the net effect of nerve activity may result from a combination of excitatory and inhibitory activity. At low frequencies of stimulation the inhibitory fibers predominate, but at frequencies above 90 cycles/sec. either the inhibitory fibers become less effective or the excitatory fibers more efficient. At a stimulation frequency of 200 cycles/sec. the excitatory fibers predominate. At the site of stimulation, the inhibitory nerves are mixed, carrying fibers which innervate leg and gill musculature as well as the cardiac ganglion. For this reason we believe that further studies of the effects of variations in the frequency of stimulation require the identification of the nerve in a region where it consists largely or exclusively of cardioregulatory fibers.

SUMMARY

1. A method of monitoring heart function in the intact *Limulus* is described.

2. Isolated hearts had rates ranging from 17 to 28, with a mean of 21.6 beats/min. The rate for any individual heart remained nearly constant ($s = 1.89$) over a period of hours.

3. Heart rate in intact animals ranged from 15 to 23, with a mean of 18.7 beats/min. The rate for any individual animal remained nearly constant ($s = 0.36$) over a period of hours.

4. Electrical stimulation of the last two pairs of nerves leaving the hindbrain causes a slowing in heart rate which is dependent on stimulus strength and frequency. Maximum slowing occurs with stimulation frequencies between 10 and 80 cycles/sec. and a lesser slowing at frequencies outside this range.

5. Changes in heart rate are not tightly coupled to stimulation of the inhibitor nerves. A time lag in response occurs both at the beginning and the end of stimulation periods.

LITERATURE CITED

CARLSON, A. J., 1905. The nature of cardiac inhibition with special reference to the heart of *Limulus*. *Amer. J. Physiol.*, **13**: 217–240.

CARLSON, A. J., 1907. On the mechanism of the stimulating action of tension on the heart (*Limulus*). *Amer. J. Physiol.*, **18**: 149–155.

CARLSON, A. J., 1909. Invertebrate hearts. *Ergebn. Physiol.*, **8**: 371–462.

CHAO, I., 1933. Action of electrolytes on the dorsal median nerve cord of the *Limulus* heart. *Biol. Bull.*, **64**: 358–382.

FLOREY, E., 1960. Studies on the nervous regulation of the heartbeat in decapod crustacea (crayfish). *J. Gen. Physiol.*, **43**: 1061–1082.

LOCHHEAD, J. H., 1950. *Xiphosura polyphemus. In:* Selected Invertebrate Types (ed. by F. A. Brown, Jr.), pp. 360–381. John Wiley and Sons, Inc., New York.

MAYNARD, D. M., 1961. Cardiac inhibition in decapod crustacea. *In:* Nervous Inhibition (ed. by E. Florey), pp. 144–178. Pergamon Press, New York.

MITCHELL, G. A. G., 1956. Cardiomuscular Innervation. E. and S. Livingston Ltd., London.

PATTEN, W., AND W. A. REDENBAUGH, 1900. Studies on *Limulus.* II. The nervous system of *Limulus polyphemus* with observations upon the general anatomy. *J. Morph.,* **16**: 99–200.

PROSSER, C. L., 1961. Circulation of body fluids. *In:* Comparative Animal Physiology, Second Ed. (ed. by C. L. Prosser and F. A. Brown, Jr.), pp. 386–416. W. B. Saunders Co., Philadelphia.

SPECTOR, W. S., 1956. Handbook of Biological Data. W. B. Saunders Co., Philadelphia.

WELSH, J. H., 1953. The action of acetylcholine antagonists on the heart of *Venus mercenaria. Brit. J. Pharmacol.,* **8**: 327–333.

WELSH, J. H., 1957. Neurohormones or transmitter agents. *In:* Recent Advances in Invertebrate Physiology (ed. by B. T. Scheer), pp. 161–171. Univ. of Oregon Publ., Eugene, Oregon.

D_2O UPTAKE RATE IN TWO BRACKISH-WATER NEREID POLYCHAETES

RALPH I. SMITH

Department of Zoology, University of California, Berkeley 4, California

In the previous study (Smith, 1963) *Nereis* (*Neanthes*) *succinea* was shown to have a higher salt-loss rate than *Nereis diversicolor* and *N. limnicola*, a difference correlated with the salinity of the natural habitats of these species. Under the standard conditions of the above experiments (a one-hour exposure to glass-distilled or deionized water following adaptation to sea water of chlorosity 10 g. Cl/L.), it was observed that *N. succinea* swelled greatly, to over 150% of its initial weight, but *N. diversicolor* swelled only to 125%, and *N. limnicola* to about 110% of initial weight. It is evident that in *N. succinea* the net water influx through the body surface is high, but the foregoing observations do not prove that *N. diversicolor* and *N. limnicola* are really less permeable to water. Quite possibly the latter two species have the same inward permeability to water (water influx per unit of surface) as *N. succinea*, but are more efficient in controlling volume by disposing of excess water as urine. It must also be considered that, although *N. diversicolor* and *N. limnicola* seem to approximate the "surface rule" in respect to salt loss as a function of size, *N. succinea* deviates in such a way as to suggest a change in shape during growth, so that the surface/volume ratio does not decrease as much as might be expected with increase in body size. *N. succinea* develops large parapodial lobes, which may be responsible for this effect. If so, the greater water intake by *N. succinea* might be the result of a relatively great surface, rather than of a higher water-permeability than is found in *N. diversicolor* and *N. limnicola*.

On the other hand, it would seem reasonable for animals living in water of low salinity to have included reduced permeability to water among their physiological or morphological adaptations although, as has been clearly shown by Shaw (1959) in the case of certain fresh-water crustaceans, the attainment of a reduced permeability to salts is not necessarily accompanied by an equally great reduction in water permeability. Since no data seem to have been published on the water-permeability of polychaetes, apart from studies of osmotic water influx, it seemed desirable to make a preliminary study of the penetration rate of "heavy water" (deuterium oxide, D_2O) into *N. succinea* and fresh-water representative of *N. limnicola*, in order to gain a general picture of the difference, if any, in the water-permeability of these euryhaline polychaetes.

METHODS

The D_2O influx was compared both under conditions of no osmotic water influx, and under conditions of hyperosmotic regulation in which there was presumed to be an inward movement of water as a result of an osmotic gradient. The worms were adapted in the laboratory for a week or more to dilutions of sea water having chlorosities of 260, 104, and 26 mM/L., hereafter referred to by their approximate values of 50%, 20%, and 5% sea water and, in the case of *N. limnicola*, to fresh water from Lake Merced in San Francisco.

Five per cent D_2O mixtures from a 93.5 mole % stock were made up in the above media for test purposes, producing a slight lowering of their salt concentration, considered negligible in terms of osmotic stress for the euryhaline species used. Worms in 50% sea water are considered to be in osmotic equilibrium with the medium but those in 20% sea water and lower concentrations are known to be regulating, and thus maintaining an osmotic gradient which presumably causes a net inward water flux through the integument. After preliminary experiments had indicated that D_2O entrance in the absence of an osmotic gradient was very rapid (half-saturation times of less than five minutes), observations were confined to five-minute exposures to 5% D_2O, in order to reduce the complications of back-diffusion of D_2O and recycling *via* the urine. Worms were blotted dry on filter paper, weighed to the nearest mg. on a torsion balance, and dropped into 10–20-ml. volumes of 5% D_2O for five minutes. Worms were removed, quickly rinsed in a small strainer by a jet from a wash-bottle, blotted, and a sample of a few drops of coelomic fluid taken in a capillary pipette for estimation of the percentage of D_2O present. Most worms of less than 100 mg. wet weight were, however, sampled entire.

For determination of D_2O concentrations, sample drops (or whole worms) were distilled in "Aloe" disposable pipettes (short style) that had been boiled in distilled water and oven-dried. A light plug of cotton was inserted past the constriction, and the sample pipetted into the space between constriction and plug, the pipette base closed by a small cork, and the tip quickly sealed in a flame. For distillation the sealed pipettes were laid on a slide-warmer set at *ca.* 60° C., and covered with a folded towel. Their tips rested on the surface of a brass block set in a pan of ice and water. Distillation was allowed to proceed to dryness overnight, so that all H_2O and D_2O of the sample were transferred to the distillate. The use of only moderate heat lessened the chance of distilling over volatile contaminants, and controls indicated that drops of known D_2 concentration could be distilled in this way with negligible change of concentration.

The D_2O concentrations of the distillates were estimated by comparison with standards made up in distilled water and introduced into a bromobenzene-kerosene density-gradient column, the method being simplified after that introduced by Linderstrøm-Lang *et al.* (1938a, 1938b). I am indebted to Dr. Grover C. Stephens for suggesting the method and for advice on preparing the gradient. Details have been prepared for use in the author's class laboratory and will be furnished upon request of anyone interested. In its unrefined form, as used here, the method has proved adequate for obtaining what appear to be significant, although not very precise, data.

OBSERVATIONS

(a) *Comparison of rates of salt loss as a function of body size.* Data on salt-loss rates in the two species used in these D_2O uptake studies are given in the previous paper (Smith, 1963), but for comparison certain data are here replotted on a weight-specific basis. In Figure 1 are shown the rates of salt-loss (in mM/mg.

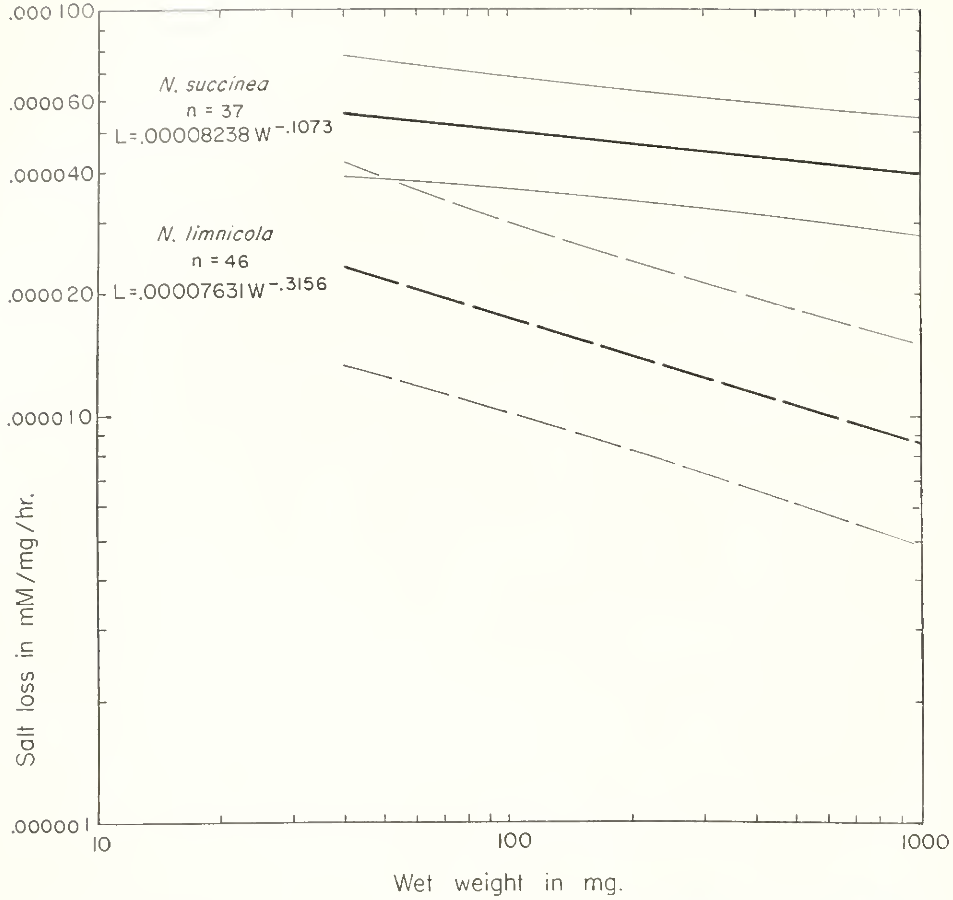


FIGURE 1. Weight-specific curves of salt-loss rate in *N. succinea* and *N. limnicola* as determined in a standard exposure to distilled water after adaptation in approximately 50% sea water; 95% confidence limits are drawn for each curve.

wet weight/hour) of worms adapted to 50% sea water and exposed for one hour in glass-distilled water. These curves are weight-specific salt-loss rate curves according to the expression:

$$\mathrm{L} = \frac{\mathrm{m}M\ \text{salt-loss/hour}}{\text{mg. wet weight}} = \mathrm{a}\ (\text{Wet Weight})^{(\mathrm{b}-1)}$$

For *N. succinea:* $\mathrm{L} = 0.00008238\mathrm{W}^{(-0.1073)}$
For *N. limnicola:* $\mathrm{L} = 0.00007631\mathrm{W}^{(-0.3156)}$

The slopes of these curves are significantly different ("t" test at 0.01 level). The slope of the curve for *N. limnicola* does not differ significantly from that characteristic of the "surface rule" ($\mathrm{b} - 1 = -0.3333$). That of *N. succinea* deviates significantly from the surface rule; this is considered a consequence of the exten-

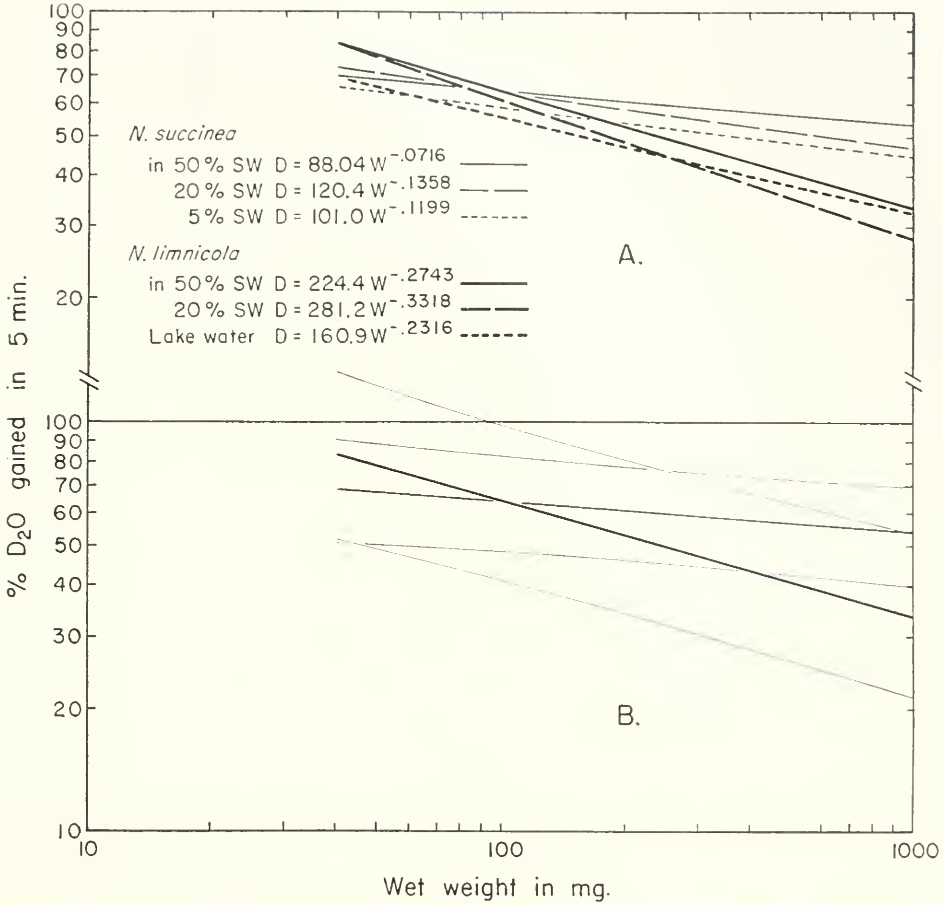


FIGURE 2. Weight-specific curves of rate of D_2O entry into *N. limnicola* and *N. succinea* in a 5-minute exposure (for details see text). Lines span the range of sizes used in the experiment. A. Curves for both species in *ca.* 50% and 20% sea water, as well as 5% sea water for *N. succinea* and lake water for *N. limnicola*. B. Curves for *ca.* 50% sea water only, replotted with 95% confidence limits drawn in.

sive flat parapodial lobes developed on older and larger members of this species, which tend to prevent much decrease in surface/volume ratio with increase of size.

The 95% confidence limits for the two salt-loss curves overlap for worms of less than 50 mg. wet weight, but over practically the whole range in which it has been possible to make such studies, it is clear that *N. succinea* loses salts at a much higher rate than *N. limnicola*.

b) *Comparison of rates of D_2O penetration as a function of body size.* In Figure 2 are shown curves for D_2O penetration in a five-minute exposure, expressing the concentration of D_2O in the coelomic fluid as a percentage of the D_2O concentration of the external medium. Several facts are apparent upon inspection of Figure 2:

(1) There is no difference in the penetration rate of D_2O into these two species, at least in the lower size range. Unlike the widely separated curves for salt-loss of *N. succinea* and *N. limnicola*, the D_2O penetration curves intersect within the size range of animals used experimentally. The permeability to D_2O is not correlated with the salt-loss tendency in these two species.

(2) It is equally clear that the groups of curves for the two species differ in slope in a characteristic way. Like the curve for salt-loss, the D_2O-penetration curves for *N. limnicola* have slopes approximating that predicted from the "surface rule"; those of *N. succinea* depart from this rule in the same sense as does the curve representing its salt-loss rate. For each species the slopes of the curves for salt-loss and D_2O-penetration do not differ significantly (t-test). Since D_2O-penetration is almost certainly *via* the body surface, the close correspondence in slope suggests that salt-loss in these two species is also, or in large part, a surface phenomenon.

(3) There is no suggestion that D_2O is entering these worms any more rapidly along an osmotic gradient than in the absence of such a gradient. In fact, the curves (Fig. 2A) for D_2O entrance in worms regulating against 20% sea water or lower concentrations suggest less entrance of D_2O. However, until a refinement of methods is achieved, it would be correct to say only that no increased entry of D_2O with an increase in osmotic gradient has been demonstrated. That the entry of D_2O may be less in lower salt concentrations is an interesting possibility, which would mean a lower water-permeability in such situations. Jørgensen and Dales (1957) have, indeed, suggested that this may be true in *Nereis diversicolor.*

(4) Figure 2B shows the extensive overlap of the 95% confidence limits for D_2O penetration rate in 50% sea water, in marked contrast to the lack of overlap for such limits in the case of salt-loss (Fig. 1). On this basis we may say that over the ordinary size range *N. succinea* shows a higher salt-loss rate than does *N. limnicola*, although these two species are equally "open" to the inward diffusion of D_2O, with or without an osmotic gradient. Although the slopes seem explicable in terms of the shapes of the worms involved, it is clear that the two species can be said to show an equal water influx at a wet weight of 100 mg. Extrapolation to smaller weights would be unreasonable. Upward extrapolation leads to an unrealizable prediction. For instance, the curves in Figure 2B intersect at a wet weight of 100 mg. and the 95% confidence limits if projected would not cease to overlap until a body weight of over 10 grams was reached (an academic point, since *N. limnicola* rarely exceeds one gram, and *N. succinea*, two grams). One can say that the deuterium oxide influx is the same in smaller members of both species, and that *N. limnicola* shows a decreasing water influx with size as a consequence of its falling surface/volume ratio. As in the case of salt-loss rates, *N. succinea* departs from this surface relationship, apparently by a change of shape during growth. The tendency of larger individuals of *N. succinea* to show a higher D_2O-influx than *N. limnicola* may be a consequence of relative surface area rather than the result of a higher water-permeability in the former species.

DISCUSSION

Although *N. limnicola* takes up less water than *N. succinea* when subjected to an equal osmotic gradient, the above observations show that both species are very "open" and about equally permeable to the inward diffusion of D_2O even in the absence of an osmotic gradient or net inward water flux. The fact that *N. limnicola* takes advantage, so to speak, of the surface rule means that large individuals of this species would take in less D_2O than equivalent specimens of *N. succinea* but, as has been pointed out, this difference is essentially only a morphological one. From the physiological point of view, the most interesting differences are the discrepancy between the observed osmotic water intake and that calculated from D_2O diffusion, and the discrepancy between the extents of osmotic swelling shown by the two species. The present data do not permit a complete treatment of this problem, but the following preliminary considerations will indicate some of the problems that must be solved in consequence of the complex nature of the "permeability" of the brackish-water nereids.

For purposes of discussion, assume that in the approximately 50% sea water used in these experiments the worms are not regulating and that the water concentration both inside and outside the worms is the same. In this osmotic equilibrium there should be an equal diffusion of water inwards and outwards, that is, there should be no net water flux in either direction. Now, in this equilibrium situation, 100-mg. specimens of both *N. limnicola* and *N. succinea* have been shown to have the same D_2O uptake, namely, 64% of the external D_2O concentration is reached by inward diffusion in five minutes (1/12 hour). Ignoring back-diffusion and other complications, and accepting a water content value of 85% of the wet weight of the worms (an approximation based on unpublished data of Mr. Larry C. Oglesby of this laboratory), and assuming that D_2O were to replace all H_2O in the external medium, the D_2O diffusional influx may be calculated as $0.64 \times 85 \times 12 = 653$ mg. of water (as D_2O) entering per hour, by diffusion. In such an hypothetical situation, the external concentration of D_2O would be 49.4 osmolar. The inward D_2O diffusion per osmole of concentration difference would be (653 mg. per hour/49.4 osmoles) = 13.2 mg. D_2O per osmole of concentration difference per hour, and this (since we are assuming that D_2O is equivalent to H_2O) would be balanced by an equal and outward diffusion of water.

If we now consider the situation of a worm pre-adapted to 50% sea water and then placed into distilled water, the water concentration inside would still be 54.7 osmolar, and that outside would be 55.4 osmolar (pure (H_2O), a concentration difference of 0.7 osmole. Associated with such an excess concentration of water outside, there should be a net inward (osmotic) flow superimposed on the diffusional exchange. If the osmotic inflow were simply diffusional, and if we accept the diffusional influx as being 13.2 mg./osmole/hour, the net inward (or osmotic) inflow is 0.7×13.2 mg./hour = 9.24 mg. hour. The figure of 9.24 mg. equals 10.8% of the body water of a 100-mg. worm, and this should represent the gain in weight taking place in one hour in distilled water, which was the standardized osmotic emergency used in the previous study (Smith, 1963). But, it is clear that the observed osmotic swelling in *N. succinea* was much greater than predicted on this basis of the D_2O diffusion rate. The agreement is, however, good in the case of *N. limnicola:*

	Observed increase in weight (% wet weight)	Observed increase as % of body water
N. limnicola	10%	11.8%
N. succinea	50%	58.8%

In view of the scatter of the data, the similarity of predicted and observed water intake of *N. limnicola* need not be stressed. But there is remarkable disagreement between predicted and observed water intake in *N. succinea*, and equally remarkable is the difference in water intake between the two species in view of their equal influxes of D_2O.

That permeability, as measured by D_2O penetration, may be quite different from that estimated by osmotic water uptake, was first observed in the frog by Hevesy, Hofer and Krogh (1935), and has more recently been studied in vertebrate epithelia and eggs by several workers, including Koefoed-Johnsen and Ussing (1953), Prescott and Zeuthen (1953), Hays and Leaf (1962). Ussing (1954) offered as an explanation the concept of a surface combining areas through which diffusion might occur, and "pores" (or areas behaving like pores) through which a bulk flow of water might take place. The present data on D_2O uptake suggest that *N. limnicola* and *N. succinea* offer equally open diffusional pathways for water entry. If this view can be accepted, then either or both of two other factors may explain the observed differences in the degree of osmotic swelling undergone by the two species.

The first possibility is that these species differ in "porosity," with *N. limnicola* having such small "pores" as to impede bulk flow of water. The second possibility is that the two species do not differ at all in "porosity" (or in the tendency to permit osmotic water entry), but differ only in their ability to eliminate excess water as urine. On the latter hypothesis, the observed difference in net or osmotic water uptake as shown by swelling would be accounted for by the expulsion as urine by a 100-mg. *N. limnicola* of 58.8–11.8%, or 47%, of 85 mg. = 40 mg. of urinary water per hour *more than* that expelled by *N. succinea*. This value of 40% of the body weight in urine per hour is high, but is based upon the conditions of an osmotic emergency plus the assumption of equal "porosity" in the two species. It would be at least as reasonable to suppose that both porosity and urinary output differences are concerned as to assume one in the absence of the other. Even if *N. limnicola* could be shown to have an output of urine as high as 40% of the body weight per hour in the stated situation, its output in the natural fresh-water habitat would not be above 20%, since in that situation its internal concentration would not approximate that of 50% sea water, but would be closer to that of 25% sea water. But, until an absolute value for the urinary output of *N. succinea* is available, the volume of urine actually produced by *N. limnicola* cannot be estimated from the present data.

The observed equality of D_2O-influx, independent of the apparent rate of osmotic water intake, suggests that *N. limnicola* has the more effective water-elimination system, which would be consistent with its demonstrably better volume control. It is further evident that the D_2O influx bears no relation to the tendency to outward passage of salts. These facts need to be considered in future studies on osmoregulation in polychaetes; their full significance must await further experimentation. At present the actual water-permeability coefficient of these nereids cannot be estimated, since we do not know the surface area of these complexly-shaped animals, nor can we assume that all parts of their surfaces are equally permeable. That vertebrate epithelia represent complex systems for penetration by water has been repeatedly indicated (references cited above), in studies that show a clear separability of diffusion permeability and osmotic (filtration) permeability. This distinction has not previously been stressed in studies on invertebrates, in which water-permeability has usually been estimated from osmotic fluxes.

SUMMARY

1. A simplified method for the estimation of heavy water (D_2O) uptake in polychaetes is presented.

2. It is shown that, although *Nereis liminicola* shows a smaller salt-loss and less net osmotic water uptake in a salt-free medium than does *N. succinea,* both species show an equal D_2O influx at a body weight of *ca.* 100 mg.

3. As in the case of salt-loss, *N. limnicola* follows the "surface rule" in its D_2O uptake: *N. succinea* does not.

4. No increased uptake of D_2O has been demonstrated in lower salinities in which there is presumed to be an osmotic inflow of water. This is consistent with the possibility that water-permeability is less at low environmental salinities.

5. A preliminary analysis of the osmotic and diffusional influxes of water in these two species suggests that their different osmoregulatory capabilities may be related to urine output (greater in *N. limnicola*), to a difference in integumental "pore size" (smaller in *N. limnicola*), or to some combination of these factors.

LITERATURE CITED

HAYS, R. M., AND A. LEAF, 1962. Studies on the movement of water through the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.*, **45**: 905–919.

HEVESY, G. V., E. HOFER AND A. KROGH, 1935. The permeability of the skin of frogs to water as determined by D_2O and H_2O. *Skand. Arch. Physiol.*, **72**: 199–214.

JØRGENSEN, C. B., AND R. P. DALES, 1957. The regulation of volume and osmotic regulation in some nereid polychaetes. *Physiol. Comp. et Oecol.*, **4**: 357–374.

KOEFOED-JOHNSEN, V., AND H. H. USSING, 1953. The contributions of diffusion and flow to the passage of D_2O through living membranes. Effect of neurohypophyseal hormone on isolated anuran skin. *Acta Physiol. Scand.*, **28**: 60–76.

LINDERSTRØM-LANG, K., O. JACOBSEN AND G. JOHANSEN, 1938a. On the measurement of the deuterium content in mixtures of H_2O and D_2O. *C. R. Lab. Carlsberg, Sér. Chim.*, **23**: 17–25.

LINDERSTRØM-LANG, K., AND H. LANZ, JR., 1938b. Studies on enzymatic histochemistry. XXIX. Dilatometric micro-estimation of peptidase activity. *C. R. Lab. Carlsberg, Sér. Chim.*, **21**: 315–388.

PRESCOTT, D. M., AND E. ZEUTHEN, 1953. Comparison of water diffusion and water filtration across cell surfaces. *Acta Physiol. Scand.*, **28**: 77–94.

SHAW, J., 1959. Salt and water balance in the East African fresh-water crab, *Potamon niloticus* (M. Edw.). *J. Exp. Biol.*, **36**: 157–176.

SMITH, R. I., 1963. A comparison of salt loss rate in three species of brackish-water nereid polychaetes. *Biol. Bull.*, **125**: 332–343.

USSING, H. H., 1954. Membrane structure as revealed by permeability studies. Pp. 33–42 in "Recent Developments in Cell Physiology," Proc. Seventh Symp. Colston Res. Soc., Academic Press, New York.

MARINE BIOLOG
WOODS HOLE
LIBRARY
MASS

UPTAKE OF ORGANIC MATERIAL BY AQUATIC INVERTEBRATES. III. UPTAKE OF GLYCINE BY BRACKISH-WATER ANNELIDS[1]

GROVER C. STEPHENS

Dept. of Zoology, University of Minnesota, Minneapolis, Minnesota 55455

In previous work (Stephens and Schinske, 1961; Stephens, 1962a; Stephens, in press) it has been shown that a number of marine invertebrates are capable of removing glucose and amino acids from dilute solution in the surrounding sea water. In the case of the maldanid worm, *Clymenella torquata* (Stephens, 1962b, 1962c), measurements were made of the rate of uptake of amino acids and compared with the level at which these compounds occurred in the habitat of the organism. These results suggested that this uptake process might contribute significantly to the nutrition of these animals.

Repeated attempts have been made in this laboratory to demonstrate uptake of amino acids and sugars in fresh-water invertebrate Metazoa. Twelve genera, representing six phyla (Table I), were examined with respect to their capacity to remove glucose and glycine from dilute solution, using C^{14}-labelled compounds. In no case could removal of more than a few per cent of these compounds in a 24-hour period be demonstrated. In a few cases where the labelled carbon was observed to decrease more rapidly in the ambient medium, addition of neomycin reduced the apparent rate of disappearance by 80% to 90%. Although it certainly cannot be maintained that any of the fresh-water forms employed are closely related to marine invertebrates previously studied, these uniformly negative results are in sharp contrast to the report of Stephens and Schinske (1961). They observed uptake of glycine by all of the genera of marine invertebrates employed (except arthropods), regardless of taxonomic position, habitat, and predominant feeding mechanisms.

TABLE I

Fresh-water organisms examined for their ability to remove glucose and glycine from solution

- Phylum Porifera
    - *Spongilla lacustris*
- Phylum Cnidaria
    - *Hydra oligactis*
- Phylum Platyhelminthes
    - *Dugesia dorotocephala*
- Phylum Ectoprocta
    - *Pectinatella magnifica*
- Phylum Annelida
    - *Lumbricus terrestris*
    - *Placobdella parasitica*
- Phylum Mollusca
    - *Physa* sp.
    - *Limnaea stagnalis*
    - *Sphaerium* sp.
    - *Pisidium* sp.
    - *Amblema costata*
    - *Eliptio dilatatus*

It was then attractive to examine an organism capable of tolerating extreme changes in salinity, in the hope of establishing some relationship between the osmotic concentration of the medium and the capacity for uptake of small organic molecules. The brackish-water nereids offer a number of advantages for such an investigation. They are capable of surviving abrupt transfers within a very broad range of salinities. These worms have served as the subject of a number of studies which have provided information concerning their distribution, their capacity for osmoregulation and for chloride regulation, and other features of their physiological ecology. A summary and review of some of this information is presented by Smith (1959a). The fact that data in the literature permit an estimate to be made of the salinity at which osmoregulation and chloride regulation become conspicuous is particularly desirable. This permits a more searching exploration of the potential relationship between uptake of small organic compounds and such processes. The present report presents the results of work carried out to investigate such a relationship in two species of nereids.

---

[1] Supported by USPHS Grant GM-06378.

MATERIAL AND METHODS

Two species of *Nereis* (*sensu lato*) were employed. *Nereis limnicola* Johnson (=*Neathes lighti* Hartman: see Smith, 1959b, concerning synonymy) was collected from Lake Merced and Walker Creek in California. The chloride content of Lake Merced was measured as 3.18 meq./liter which is a salinity of approximately 0.216‰. This is then a fresh-water habitat according to the Venice System of classification (Caspers, 1959). At the time of collection at Walker Creek, the chloride content of the surface water was 72 meq./liter and the interstitial water was 123 meq./liter, a mixohaline environment in an estuarine situation where considerable fluctuations would be expected due to the tidal cycle.

The second species employed was *Nereis succinea* Frey and Leuckart (= *Neathes succinea*).[2] Collections were made in San Francisco Bay, at chloride concentrations of 285 to 405 meq./liter (approximate salinities, 18.00‰ to 25.50‰), the mixopolyhaline zone in the Venice System.

Animals were collected as required and maintained in the laboratory in large fingerbowls. About a centimeter of sand from the collection site was provided as a substrate covered by one to two centimeters of sea water or sea water diluted with pond water to provide lower salinities. Animals which did not burrow in the substrate were not used nor were animals used within 48 hours of collection. The exception to this last statement is the set of observations dealing with the change in rate of uptake with time where measurements were made 24 hours after collection of *Nereis limnicola*. Animals subjected to changes in salinity in the laboratory were allowed at least 48 hours to accommodate to the change.

In determining the uptake of glycine, the following procedure was used. Dilutions of sea water were prepared, using glass-distilled water, to give the same chloride concentration as the medium in which the animals were being maintained. Measured amounts of glycine-2-C^{14} were added to this diluted sea water. Groups of animals were then placed in a suitable volume of this solution for 15 minutes to one hour. After this exposure, the animals were rinsed in diluted sea water of the same chloride content and weighed to the nearest milligram. They were then extracted at room temperature in 2.0 ml. of 80% ethanol (5.0 ml. in some cases) for 24 hours. Radioactivity of the alcohol extract was determined by evaporating duplicate 0.5-ml. samples on planchets and counting with a thin window Geiger-Muller detector. In some cases, the animals were extracted for an additional 24 hours with 5 to 10 ml. of 80% ethanol, breis prepared in a measured volume of distilled water, using a tissue homogenizer, and the radioactivity of the alcohol-insoluble fraction determined. Initial and final measurements were made of the radioactivity of the medium. In all cases, the data have been corrected for background and sample thickness.

---

[2] *Nereis succinea* Frey and Leuckart is considered conspecific with *Nereis limbata* Ehlers of the Woods Hole region.

Chloride concentrations were measured electrometrically with a chloridometer in samples taken at the time of collections. Media of chloride content lower than sea water were prepared volumetrically from sea water of known chlorosity.

RESULTS

Observations on *Nereis limnicola* will be reported first and *Nereis succinea* introduced later for comparative purposes.

Twenty-two *N. limnicola* collected at Lake Merced were acclimated for four days to sea water at a concentration of 543 meq. Cl^-/liter. They were then placed in 100 ml. of sea water at this concentration containing 0.15 mg. glycine-2-C^{14} (2×10^{-5} M/l., 400 cpm.). After one hour the animals were rinsed in sea water, weighed, and placed individually in 2.0 ml. of 80% ethanol. The radioactivity (2000 counts, background approximately 19 cpm) in the alcohol extract was measured 24 hours later. A regression line was calculated for the log of cpm in 0.5 ml. of the alcohol extract (this ranged from 52 to 282 cpm in these observations) as a function of the log of wet weight. The slope obtained was 0.483 ± 0.052. By making suitable corrections for the absorption of the samples, the ratio of alcohol-soluble radioactivity per unit volume to ambient radioactivity could be calculated. This accumulation ratio ranged from 0.75 to 2.72 and was negatively correlated with weight, as would be expected on the basis of the regression line reported above. An alternative way of expressing the rate of uptake for such a group of animals is used in much of the data to be reported. The radioactivity of the alcohol extract is divided by the square root of the wet weight (as an acceptable and convenient approximation of the exponent, 0.483, relating uptake and weight). Uptake can then be calculated for a selected weight. The animals employed in these observations ranged in weight from 56 to 472 mg. and averaged approximately 200 mg. The arbitrary factor representing uptake for this group is 7.88 ± 2.35. Suitable calculations indicate an accumulation ratio of 1.75 for a 200-mg. animal. Alternately, one may calculate that an average 200-mg. animal acquired 0.53 ± 0.16 micrograms of glycine which remained in alcohol-soluble form. The justification for referring to the ratio of alcohol-soluble radioactivity to ambient activity as an accumulation ratio comes from chromatography of the alcohol-soluble material. The radioactivity resides in glycine in the extract.

In another group of animals, individuals were removed at 30 minutes and at 60 minutes. Radioactivity of the extract divided by the square root of wet weight was, respectively, 22.7 ± 5.9 ($n = 9$) and 45.5 ± 17.6 ($n = 9$). Hence the process is linear with time for at least 60 minutes.

Groups of animals were tested for their capacity to remove glycine from ambient sea water at various chlorosities. The worms were tested three or four days after they were placed in water of the designated concentration. In order to compensate for differences in the ambient glycine-2-C^{14}, which ranged from 10^{-5} to 3×10^{-5} M/l., uptake is expressed in arbitrary units derived as follows. The

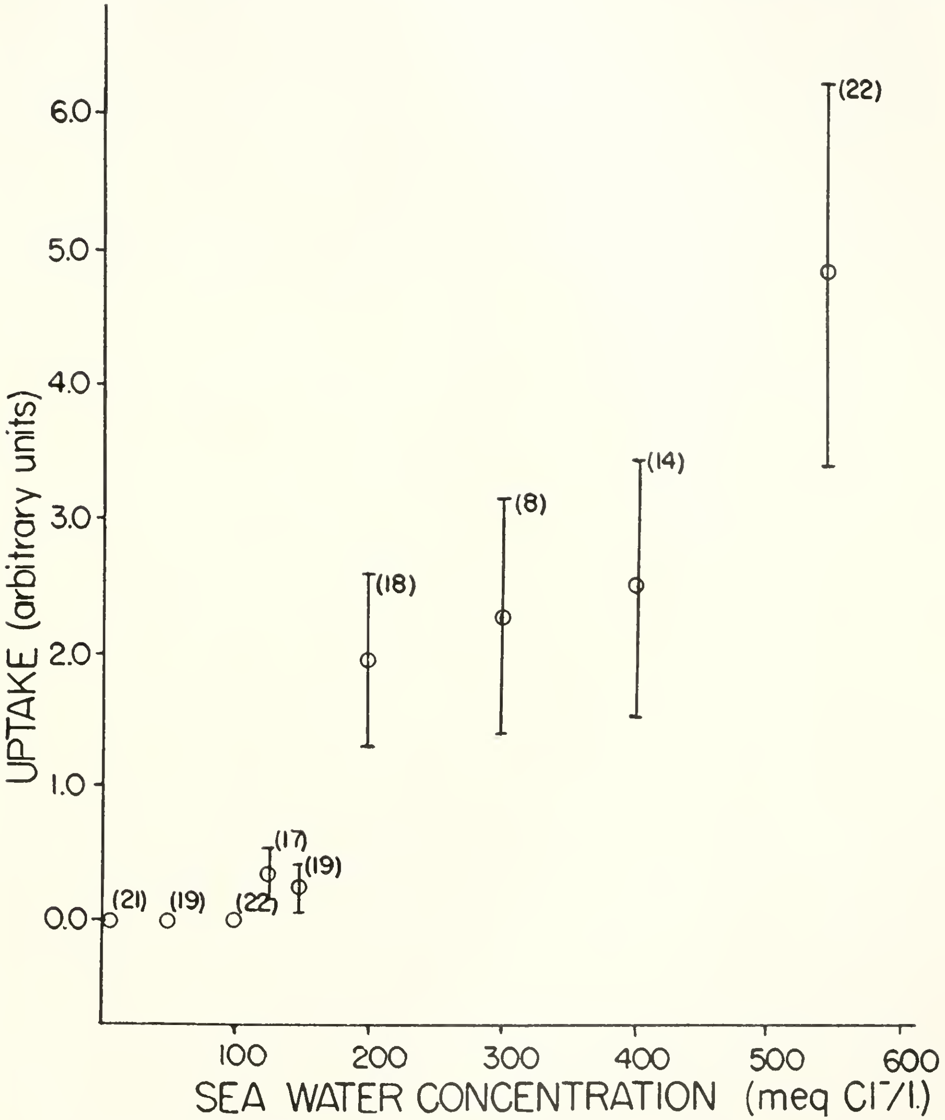


FIGURE 1. Rate of uptake of glycine by *Nereis limnicola* as a function of the chlorosity of the ambient medium. The bars represent standard deviations, and the numbers in parentheses are the number of individuals observed. Uptake is in arbitrary units (see text) and has been corrected for the weight of the animals and for differences in ambient radioactivity of the solutions.

radioactivity of 0.5 ml. of the ethanol extract was divided by the square root of the wet weight and this was in turn divided by the initial ambient radioactivity. The result was multiplied by 1000. Figure 1 presents the results of such observations. Standard deviations are not presented for sea water concentrations of 3, 50 and 100 meq. Cl^-/l. since none of the radioactivities of the alcohol extracts was significantly different from background. After an additional extraction with 80% alcohol, breis of the animals were prepared and alcohol-insoluble radioactivity determined. Values ranged from 48% to 71% of the alcohol-soluble radioactivity and were not related to ambient concentration of sea water. No significant alcohol-insoluble activity could be demonstrated below 125 meq. Cl^-/l.

In the course of these observations, a group of animals which had been maintained in sea water at a concentration of 300 meq. Cl^-/l. for a period of 63 days was employed. These animals exhibited a rate of uptake which was 9.7 times greater than that presented in Figure 1 at this salinity. This suggested that the adaptation of this process to salinity change might occur much more slowly than had been assumed. To test this, worms were collected from Lake Merced and placed in sea water at 300 meq. Cl^-/l., and their capacity for glycine uptake checked periodically. Table II presents uptake in arbitrary units (corrected for weight and ambient concentration) at various times after the animals were placed at this concentration. It is clear that an approximately ten-fold increase in the rate of glycine uptake has occurred during the two weeks the process was followed, and that the adaptive capacity of the system had not necessarily been exhausted at the end of this period.

TABLE II

Mean uptake and standard deviation for groups of animals maintained at 300 meq Cl^-/l. for the time listed. Data have been corrected for variation in weight and ambient radioactivity. Ambient glycine concentrations range from 1 to 3 × 10^{-5} molar

Animals collected at Lake Merced

Days	Uptake*
1	1.74 ± 0.93 (n = 12)
4	3.16 ± 0.77 (n = 12)
7	6.87 ± 3.90 (n = 12)
11	14.55 ± 5.06 (n = 11)
14	17.75 ± 9.25 (n = 12)

Animals collected at Walker Creek

Days	Uptake*
1	7.84 ± 3.13 (n = 12)
4	14.70 ± 5.21 (n = 12)

* Uptake is expressed in arbitrary units. See text.

It was desirable to look at rates of glycine uptake in worms collected at Walker Creek since they represent an estuarine population and might be expected to be exposed to higher salinities than would be the case in the fresh-water environment of Lake Merced. As indicated in Table II, the Walker Creek animals did indeed exhibit a higher initial rate of uptake at the test salinity. They also showed an increase in rate after acclimating for four days. Unfortunately, neither population could be followed beyond the times indicated in Table II. It is perhaps worthy of comment that a population of worms kept at 50 meq. Cl^-/l. for three weeks showed no demonstrable uptake of glycine at the end of this period.

It was of interest to examine the relationship between ambient glycine concentration and the rate of uptake. Concentration curves were determined for groups of animals at sea water concentrations of 200, 300, and 520 meq. Cl^-/l. The animals in the first and third groups were collected at Lake Merced while the animals measured at 300 meq. were collected from Walker Creek. They had been maintained in the laboratory at the chlorosities indicated for 4, 4, and 6 days, respectively. Glycine concentrations ranged from 2×10^{-5} to 10^{-3} moles per liter for each group. A plot of the reciprocal of ambient glycine concentration against the reciprocal of rate of uptake gave a reasonable fit to a straight line. Correlation coefficients for the least squares regression lines were 0.74 ($n = 22$), 0.62 ($n = 36$), and 0.67 ($n = 23$) for the three groups. This procedure is equivalent to a Burk-Lineweaver plot in enzyme kinetics. Obtaining a straight line suggests that an adsorptive step becomes rate-limiting as ambient glycine concentration is increased. By analogy with an enzyme-catalyzed reaction, it is possible to calculate the apparent maximum velocity of uptake (V_{max}) from the intercept of such a plot and to calculate the concentration at which velocity is half maximal (K_m) from the intercept and the slope of the regression line. These figures and their standard deviations are listed in Table III. The V_{max} for the 200-meq. group is significantly below that for the other groups, which do not differ significantly. The K_m for the 200-meq. group is also significantly higher than that for the other groups. The data have been corrected for the size of the worms and rates are expressed on the basis of averages for 200-mg. animals. A detailed interpretation of the apparent differences would require postulating a mechanism for uptake which would permit identification of the analogues corresponding to changes in enzyme concentration, turnover number, and the complex of rate constants expressed in the K_m. This does not seem justifiable. Hence, it can simply be pointed out that the differences in rate which are apparent at low ambient concentrations are also manifest as differences in maximum velocity. The data indicate that this change is not produced merely by an increase in the number of uptake sites available since this would not modify the K_m.

TABLE III

V_{max} and K_m values with standard deviations for three groups of Nereis limnicola. *The groups are identified by the sea water concentration at which they were maintained, expressed as meq. Cl^-/l., and by their place of collection. Units for V_{max} are moles $\times 10^{-9}$ per 200-mg. worm per hour. Units for K_m are moles $\times 10^{-5}$ glycine per liter*

Group	V_{max}	K_m
200 meq. (Merced)	4.07 ± 1.12	15.0 ± 3.0
300 meq. (Walker)	22.7 ± 2.5	3.8 ± 0.9
520 meq. (Merced)	24.7 ± 6.9	3.7 ± 1.9

The facts that the relation between weight and uptake is an exponential one, and that the rate of uptake varies with ambient glycine concentration in the fashion described, suggest that uptake might be occurring directly across the surface of the animals without the necessary involvement of the gut. More direct evidence can be presented for this position. A group of animals ($n = 9$) ligated with thread at approximately the tenth setiger was compared with a control group ($n = 9$). Uptake of glycine in the two groups in arbitrary units was 10.69 ± 4.85 and 11.84 ± 4.54, respectively. When a group of worms ($n = 8$) which had been decapitated 24 hours before testing was compared with a control group ($n = 12$), uptake was 4.02 ± 1.01 and 4.17 ± 1.01, respectively. The control groups are not comparable. These procedures do not exclude the possibility of uptake *via* the posterior gut but serve to strengthen the suggestion that uptake occurs across the body wall, as

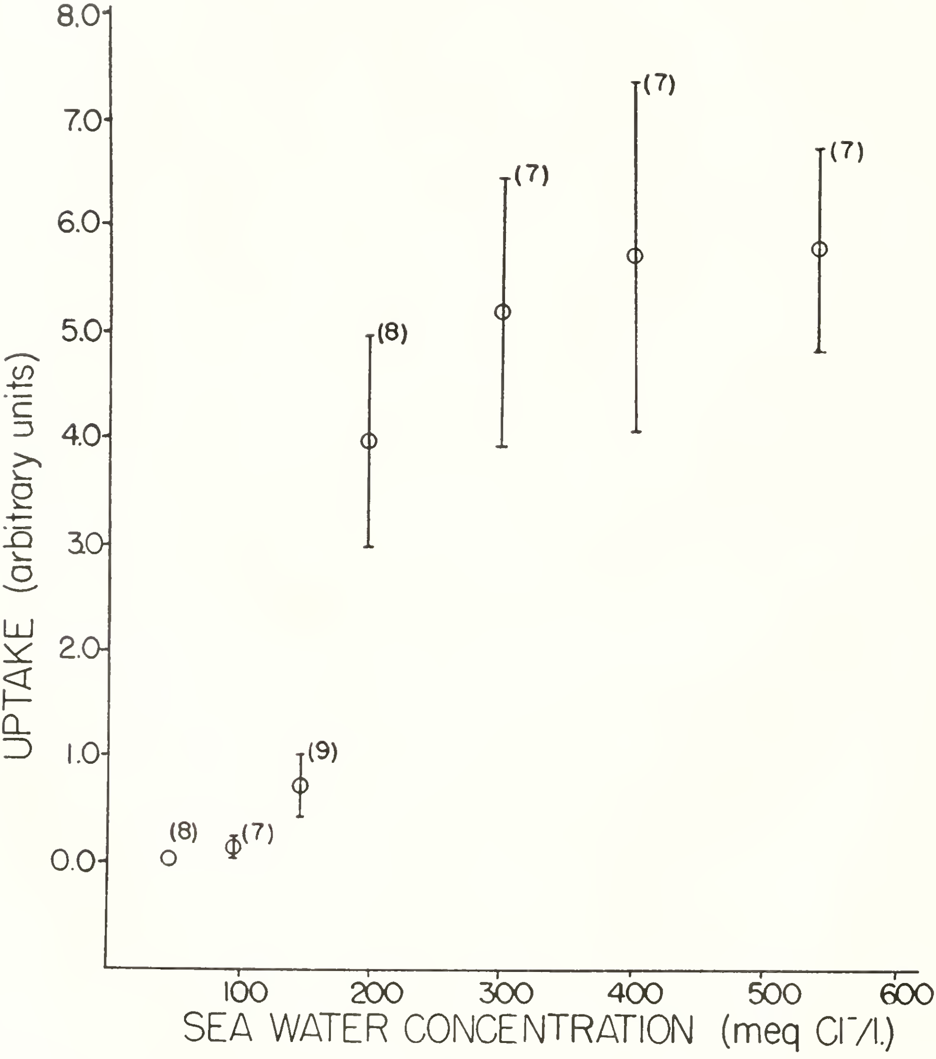


FIGURE 2. Rate of uptake of glycine by *Nereis succinea* as a function of the chlorosity of the ambient medium. The bars represent standard deviations, and the number of individuals observed is indicated in the parentheses. Uptake has been corrected for weight and for differences in ambient radioactivity.

has been reported for the maldanid annelid, *Clymenella torquata* (Stephens, in press).

Comparable observations were made on groups of *Nereis succinea*. The data present some striking similarities but also indicate significant differences between the two species. Figure 2 indicates the results of observations testing the relation between sea water concentration and the rate of uptake. The arbitrary units in which uptake is expressed are not the same as those presented for *Nereis limnicola*. In fact the rate of uptake for *N. succinea* is strikingly greater. After 15 minutes' exposure to an ambient glycine concentration of 2.7×10^{-5} M/l. in sea water at 520 meq. Cl^-/l., the ratio of alcohol-soluble radioactivity in the body water of the worms to the ambient radioactivity was 9.8 ± 2.4. This is the mean figure for a 200-mg. worm and is comparable to the data for *N. limnicola*. Thus, accumulation is proceeding at approximately 22 times the rate observed for *N. limnicola*. Another distinction between the animals is the absence of any indication of an influence of chloride concentration on uptake of glycine above 200 meq. Cl^-/l.

TABLE IV

Mean uptake and standard deviation for groups exposed to glycine at approximately 2×10^{-5} moles per liter in solutions of the compounds listed. All solutions are approximately equivalent in osmotic concentration to sea water at 200 meq. Cl^-/l.

Solution	Uptake*	% uptake in sea water
Nereis succinea		
sea water	180 ± 42	100%
NaCl	122 ± 32	68%
galactose	30 ± 7	17%
Nereis limnicola		
sea water	5.9 ± 2.8	100%
NaCl	2.6 ± 0.9	44%
galactose	1.1 ± 0.6	19%

* Uptake is expressed in the same arbitrary units used in Table II.

The greater rate of glycine uptake exhibited by *N. succinea* is also apparent in the maximum velocity of uptake estimated from a Burk-Lineweaver plot of data relating ambient concentration and uptake. As in the case of *N. limnicola*, a straight line is obtained from such a plot. The maximum velocity calculated from the regression line at 200 meq. Cl^-/liter is $287 \pm 59 \times 10^{-9}$ moles per 200-mg. worm per hour. The K_m is $2.24 \pm 0.94 \times 10^{-4}$ moles glycine per liter. Comparable figures at 520 meq. Cl^-/l. are $322 \pm 59 \times 10^{-9}$ moles per worm per hour with a K_m of $1.13 \pm 0.47 \times 10^{-4}$ moles glycine per liter. The differences in the two groups are not significant.

It is apparent from Figures 1 and 2 that in the case of both animals, a sharp decrease in rate of uptake of glycine occurs between 200 and 150 meq. Cl^-/l. and that uptake has entirely or virtually stopped at 100 meq. A series of observations was undertaken to determine whether glycine uptake would proceed in other solutions whose osmotic concentration was approximately that of sea water at 200 meq. Cl^-/l. Solutions of galactose (400 mM/l.) and sodium chloride (200 mM/l.) were employed. Galactose was used since *N. limnicola* was observed to take up glucose from dilute solutions. Although galactose was not directly checked as a possible substrate for uptake, previous work (Stephens, 1962a, and unpublished observations) indicated that galactose is not removed from solution by the coral, *Fungia,* or the clam, *Mercenaria mercenaria.* This of course does not exclude the possibility that it may be taken up by nereids. Table IV presents uptake in arbitrary units from the ambient solutions listed. In each case, glycine concentration was approximately 2×10^{-5} M/l. Worms of both species survived such exposures but were clearly not normal in their behavior. The worms in galactose became quite sluggish, while those in sodium chloride collected in a tangled mass in a fashion which never occurred at any concentration of sea water. Hence, the decrease in rate of uptake in galactose and sodium chloride cannot be referred to specific ion effects with any confidence, since toxic effects of the solutions are probable. One may conclude that provided the osmotic concentration of the medium is raised to this level, at least some uptake occurs.

Discussion

Both species of nereids employed in these observations are capable of removing glycine from dilute solution in the ambient medium at intermediate and high salinities. Both species fail to exhibit this capacity at sea water concentrations below 50 meq. Cl^-/l. In both cases, a dramatic reduction in rate of uptake occurs between 200 and 150 meq. Cl^-/l. It is of interest to examine the available data concerning osmotic regulation and chloride regulation in these forms. Smith (1959a) reports data for chloride regulation and for osmotic regulation in *Nereis limnicola.* His figures can be interpreted as indicating the onset of osmotic regulation at ambient concentrations of approximately 175 meq. Cl^-/l., although measurements were not made at concentrations just greater than this figure. Hence it is more accurate to say that this is the level at which internal osmotic concentration is maintained at ambient chlorosities between 30 and 150 meq. At 300 meq. there is no significant difference between internal and external osmotic concentrations. The data for chloride regulation indicate slightly higher regulated levels (approximately 210 meq.) with a similar ambiguity concerning the point at which regulation intervenes. Oglesby (unpublished) has kindly permitted the use of data concerning osmotic regulation in *Nereis succinea,* which are summarized in Figure 3. Freezing point depression of the ambient medium and of the coelomic fluid was measured. Regression lines from data at chlorosities greater than 265 meq. and data between 54 and 150 meq. were prepared. These lines intersect at approximately 225 meq. Cl^-/l. which may be taken as an estimate of the point of onset of osmotic regulation. It is apparent that the concentrations of the external medium at which osmotic regulation begins, at which chloride regulation commences, and at which there is a sharp modification of glycine accumulation correspond quite closely.

On the basis of this information, it can be suggested that the processes which underlie osmotic regulation are incompatible with the accumulation of amino acids from the ambient medium. In further support of this possibility, the following remarks may be offered. Essentially all the marine invertebrates examined (with the exception of marine arthropods), a total of approximately 40 genera in 10 phyla (Stephens and Schinske, 1961; Stephens, 1962a, 1962b, unpublished observations), are capable of removing amino acids from dilute solution in sea water. As pointed out in the introduction to this work, none of 12 genera of soft-bodied fresh-water invertebrates in 6 phyla show this capacity. The ability of the nereids studied in the present report to take up glycine from solutions of galactose and sodium chloride at suitable osmotic concentrations is also suggestive.

With respect to the rate at which glycine is acquired by the two species of nereids, the rate exhibited by *Nereis succinea* is of the same order of magnitude as

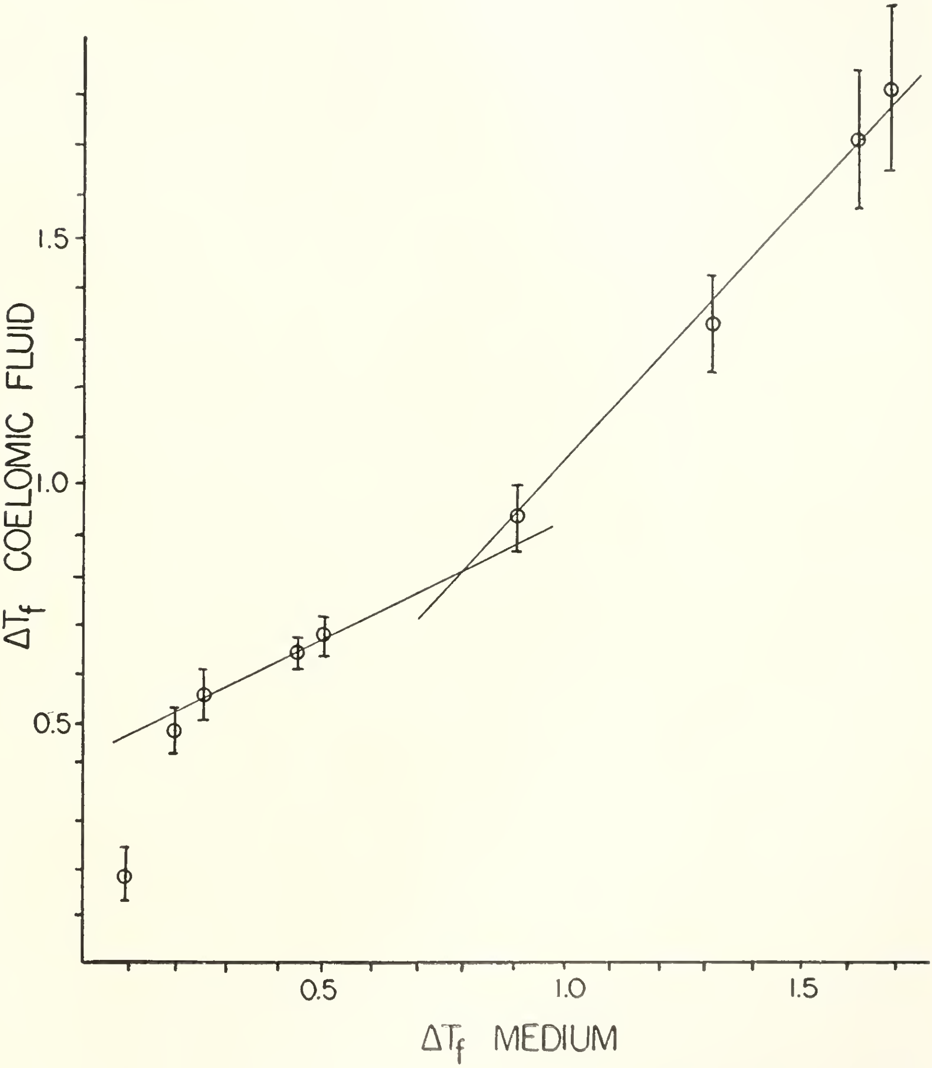


FIGURE 3. Freezing point depression of the coelomic fluid of *Nereis succinea* as a function of the freezing point depression of the medium. The bars represent standard deviations, and the lines are calculated regression lines (see text). Data furnished by L. C. Oglesby.

that exhibited by the marine worm, *Clymenella torquata* (Stephens, 1962b). Assuming that the metabolic rate of *Nereis succinea* is not grossly different from that of *Clymenella*, this implies that amounts of reduced carbon which are significant when compared with those required to support the oxygen consumption of the animal may be available by this pathway. This involves the further assumption that free amino acids are available in the environment. This appears to be the case in mud flats previously examined (Stephens, 1962c) and in other marine sediments (Belser, 1959, 1963) but has not been demonstrated in the brackish-water flats of San Francisco Bay where these animals were obtained.

By contrast, the rates of uptake exhibited by *Nereis limnicola* are an order of magnitude lower and it is difficult to imagine that this process could provide more than a small percentage of the carbon required by the animals. Furthermore, the accumulation system would only function in estuarine populations where salinity rose to the range which is permissive with respect to accumulation.

It is interesting to contrast the distribution and the ability of the two worms to regulate at extremely low salinities. *N. limnicola* tolerates exposure to distilled water and is found in a true fresh-water habitat. Figure 3 suggests that the ability of *N. succinea* to regulate osmotic concentration of the coelomic fluid may decline at very low ambient concentrations. The two species seem not to overlap in distribution. *N. succinea* replaces *N. limnicola* in brackish waters of higher salinity. The data concerning distribution and osmoregulatory ability are consistent with the position that osmoregulation and accumulation of amino acids are not compatible. They are also consistent with the speculation that the ability to accumulate amino acids may confer an adaptive advantage on organisms which inhabit marine or the more saline brackish-water habitats.

It is apparent that the process of adaptation to a change in salinity extends for a period of more than two weeks in *Nereis limnicola*. For this reason, the relation which is suggested by the data presented in Figure 1, relating uptake and ambient salinity between 200 and 543 meq. Cl^-/l., should be considered tentative. In no case had the animals been maintained for sufficient time to consider that they had adapted to the sea water concentration at which the measurements were made. No evidence was found to suggest a prolonged adaptation period to salinity change in *N. succinea*. However, the critical procedure of studying worms which had been adapted to a low salinity and then returned to a high salinity was not carried out.

The observations reported here do not bear on the potential function of amino acids which the worms may acquire by this pathway. Presumably they would enter the free amino acid pool of the organisms and hence participate in a variety of oxidation and synthesis pathways. Some evidence has been presented that this is the case in other marine worms (Stephens, in press).

I wish to express my thanks to Prof. R. I. Smith of the Zoology Department at the University of California at Berkeley for his kindness in making space available to me in his laboratory for this work. I must also acknowledge his generous help and advice in collecting and maintaining the animals employed. I would also like to express my appreciation to L. C. Oglesby of the same department for permitting me to use his unpublished data concerning osmoregulation in *Nereis succinea*.

SUMMARY

1. Both *Nereis limnicola* and *Nereis succinea* are capable of removing glycine from dilute solution in the surrounding medium at intermediate and high salinities. Uptake declines rapidly at sea water concentrations of chlorosity less than 200 meq./l.

2. The uptake is linear with time for periods of at least one hour, and appears to take place across the body wall without the necessary participation of the gut. Uptake is an exponential function of wet weight.

3. The relation between ambient concentration of glycine and rate of uptake suggests that an adsorptive step becomes limiting in the process of uptake at high concentrations of glycine. A plot of the reciprocal of uptake *versus* the reciprocal of ambient glycine concentration (Burk-Lineweaver plot) is linear and permits estimation of the apparent V_{max} and K_m for the process.

4. In *N. limnicola*, the adaptation of the physiological system mediating glycine uptake to a change in salinity is a process extending for more than 14 days.

5. Conditions which produce an increase in uptake of glycine at low ambient concentrations in *N. limnicola* also produce a significant increase in estimated maximum velocity and a decrease in the estimated K_m from a Burk-Lineweaver plot. This suggests that the increase reflects a change in the process which cannot be explained as merely an increase in the number of available sites for uptake, since this would not influence the K_m.

6. Both species are capable of removing glycine from solutions of galactose and of sodium chloride whose osmotic concentrations are approximately equal to that of sea water at 200 meq. Cl^-/l., although the rates are significantly below those observed in sea water.

7. The rate of uptake observed in *N. succinea* exceeds that in *N. limnicola* by an order of magnitude. This is correlated with the distribution of the latter species, which occurs at lower salinities, and with its greater osmoregulatory ability at low concentrations of the medium.

8. The salinity of the medium at which the process of glycine uptake declines agrees closely with estimates of the point of onset of osmoregulation and of chloride regulation in the two worms. This, together with the widespread ability of marine invertebrates to remove amino acids from solution and the failure to demonstrate this capacity for fresh-water forms, leads to the suggestion that the processes of glycine uptake and osmoregulation are incompatible.

LITERATURE CITED

BELSER, W. L., 1959. Bioassay of organic micronutrients in the sea. *Proc. Nat. Acad. Sci.*, **45**: 1533–1542.

BELSER, W. L., 1963. Bioassay of trace substances. *In:* The Sea, Ideas and Observations, Goldberg *et al.*, Ed., Wiley, New York. Vol. 2: 220–231.

CASPERS, H., 1959. Vorschläge einer Brackwassernomenklatur ("The Venice System"). *Intern. Revue ges. Hydrobiol.*, **44**: 313–316.

SMITH, R. I., 1959a. Physiological and ecological problems of brackish waters. Marine Biology, Proc. Biology Colloquium, Oregon State College, pp. 59–69.

SMITH, R. I., 1959b. The synonymy of the viviparous polychaete *Neanthes lighti* Hartman (1938) with *Nereis limnicola* Johnson (1903). *Pacific Sci.*, **13**: 349–350.

STEPHENS, G. C., AND R. A. SCHINSKE, 1961. Uptake of amino acids by marine invertebrates. *Limnol. and Oceanog.*, **6**: 175–181.

STEPHENS, G. C., 1962a. Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria*. *Biol. Bull.*, **123**: 648–659.

STEPHENS, G. C., 1962b. Uptake of amino acids by the bamboo worm, *Clymenella torquata*. *Biol. Bull.*, **123**: 512.

STEPHENS, G. C., 1962c. Amino acids in the economy of the bamboo worm, *Clymenella torquata*. *Biol. Bull.*, **123**: 512.

STEPHENS, G. C., (in press). Uptake of organic material by aquatic invertebrates. II. Accumulation of amino acids by the bamboo worm, *Clymenella torquata*. *Comp. Biochem. and Physiol.*

THE MORPHOLOGY, LIFE-HISTORY, AND SYSTEMATICS OF THE DIGENETIC TREMATODE, HOMALOMETRON PALLIDUM STAFFORD, 1904[1]

HORACE W. STUNKARD

The American Museum of Natural History, New York 24, New York

Historical Background for the Investigation

The genus *Homalometron* was erected by Stafford (1904) to contain a new species, *H. pallidum,* from the intestine of *Fundulus heteroclitus.* There was no description or figure; instead, the new species was based on the description and figure by Linton (1901) of specimens collected from *F. heteroclitus* in the region of Woods Hole, Massachusetts. The species was not named by Linton whose account (p. 442) reads, "*Distomum* sp. (Pl. XXXII, Fig. 354) Aug. 7, 1899; 12. Aug. 28, 1899; 4. Intestine. Body very minutely spinose, white, translucent; acetabulum and oral sucker about same size; outline of body, long oval; neck short, continuous with body; greatest breadth in region of testes, near posterior end; ecaudate; acetabulum sessile; rami of intestines simple, elongate; esophagus as long as pharynx; testes, two, in median line behind uterus; seminal vesicle dorsal to ovary and posterior border of acetabulum; ovary between acetabulum and testes, on right side; pharynx, subglobular; genital aperture in front of acetabulum, on median line; vitelline glands lying at posterior end and along sides of body as far as acetabulum; ova few, relatively large. Dimensions of specimen in formalin, given in millimeters: Length, 2.72; breadth, anterior 0.43, at acetabulum 0.89, middle 1.07; near posterior 0.36; diameter of oral sucker, 0.26; diameter of acetabulum, 0.29; diameter of ovary, 0.21; diameter of testes, 0.33 and 0.39; ova, 0.11 and 0.07 in the two principal diameters."

The report of Stafford was criticized by Looss (1907) who deplored the naming of species which can not be recognized from their descriptions. He stated (p. 613), "Ich verweise als Beispiel hier auf einen neueren Artikel von Stafford (1904). In demselben wird eine ganze Anzahl von Gattungen und Arten neu benannt; unter ihnen finden sich solche, wie z. B. #14) *Xenodistomum melanocystis.*" After listing the diagnostic characters, Looss continued, "Es wird aber niemand behaupten können, dass mit der Veröffentlichung dem Leser auch nur ein leiser Fingerzeig über die wahre Natur des Genus und der Species gegeben ist. Die Namen sind leere Worte und trotzdem prioritätsrechtlich unantastbar." He added (p. 614), "Noch ein andres Beispiel: #32) '*Homalometron pallidum.* Stomach and duodenum of *Fundulus heteroclitus* L. (Killifish). New genus, new species . . . (Namenserklärung) . . . Linton, Bull. U. S. Fish Commiss. 1899. (1901) p. 422. Pl. 32. f. 354." Die zitierte Figur bei Linton, wenig Information gebend, wie leider die meisten dieses Autors, erinnert an ein *Allocreadium,* zeigt

---

[1] This investigation was supported by Grant-NSF-G23561.

aber eine bestachelte Haut, würde also auf ein Mitglied der Gattung *Lepocreadium* Stoss. hinweisen."

Manter (1926) reported *Homalometron pallidum* in *Fundulus heteroclitus* from the coast of Maine. He noted the similarity to *Lepocreadium* and stated (p. 87), "So far as the description of Linton shows, the form could easily belong to this genus, the chief point of difference being the median location of the genital pore instead of a position to the left, as in *Lepocreadium*. The pore was clearly median in my material." But in addition to the difference in location of the genital pore, Manter noted that in *Lepocreadium* there is a prominent cirrus-sac which encloses the prostate gland and the anterior portion of a bipartite seminal vesicle, whereas in the species from *Fundulus* there is no cirrus-sac; the seminal vesicle is globular, not divided; and the duct from the vesicle is enclosed by glandular cells which lie free in the parenchyma. Moreover, the male duct is joined by the vagina to form a long, genital sinus. According to Manter, these features clearly distinguish *Homalometron* from *Lepocreadium* and *Homalometron* does not agree with the diagnosis of the subfamily Lepocreadiinae Odhner, 1905.

A timely and significant observation was made by Manter on the life-history of the species. He found encysted metacercariae in the stomach of *Fundulus* and very young worms, sometimes in large numbers, in the intestinal folds of the host. He concluded (p. 87) "As the Fundulus had been in salt water aquaria for some time, the indication is that infection is derived from marine rather than fresh water sources. In spite of the fact that immature specimens were sometimes found in large numbers, infection with the adult form was never heavy, and many uninfected fish were found. Usually not more than two or three specimens were taken from one host."

Looss (1894) described *Distomum isoporum* from the intestine of *Cyprinus carpio* and other fishes and he (1899) designated *D. isoporum* as type of a new genus, *Creadium*, and a new subfamily, Creadiinae. But the name *Creadium* was preoccupied and for it Looss (1900) substituted the name *Allocreadium*, whereby *ipso facto* the species, *isoporum*, remained type of the genus and subfamily. Looss (1902) named the subfamily Allocreadiinae and Stossich (1903) erected the family Allocreadiidae.

Meanwhile MacCallum (1895) described a distome from *Aplodinotus grunniens*, *Lepomis gibbosus*, and *Acipenser rubicundus* taken from Lake Erie and the Grand River of Ontario, as *Allocreadium* Looss var. *armatum*. Wallin (1909) described a new species, *Allocreadium lobatum*, with a history and revision of the genus *Allocreadium*. He noted that Looss (1902: 785) had pointed out that the species described by MacCallum is not a member of *Allocreadium*. However, Pearse (1924) identified specimens from *Aplodinotus grunniens* taken in Lake Pepin and Lake Michigan as *Allocreadium armatum* (MacCallum). Simer (1929) studied trematodes of fishes taken from the lower Tallahatchie River in Mississippi. In *A. grunniens* and *Polyodon spathula* he found the species described by MacCallum, common in smaller fishes and fewer in larger fishes. For it he erected a new genus, *Anallocreadium*. He stated that Looss had excluded the form from the Allocreadiinae; the absence of a cirrus-sac excludes it from the Lepocreadiinae; and the absence of oral papillae excludes it from the Stephanophialinae; but no final allocation was offered. Hunter and Bangham (1932) described *Anallocreadium* *pearsei* from *A. grunniens* and erected the subfamily Anallocreadiinae, which they included in the family Allocreadiidae. Manter (1936) erected the new genus, *Crassicutis*, and stated (p. 35), "The genera *Anallocreadium* and *Crassicutis* show affinities to the Lepocreadiinae through the genus *Homalometron*. . . . Further evidence of relationship to the Lepocreadiinae is to be sought in life history studies." Cable and Hunninen (1942) worked out the life-cycle of *Deropristis inflata* and discussed its systematic position. They showed that *Deropristis* can not be retained in the family Acanthocolpidae and that its nearest relatives are such forms as *Homalometron*, *Anallocreadium*, *Microcreadium*, and *Lepocreadium*. Accordingly, they accepted the Lepocreadiidae, a family erected by Nicoll [2] to contain the Lepocreadiinae Odhner and a group of similar and presumably related genera. In the family, Cable and Hunninen recognized three subfamilies: Lepocreadiinae Odhner, 1905; Deropristinae n. subfam.; and Homalometrinae (Anallocreadiinae Hunter and Bangham, 1932, renamed). In the Homalometrinae they included *Homalometron* Stafford, 1904; *Microcreadium* Simer, 1929; *Crassicustis* Manter, 1940; and an unnamed genus reported by Manter (1941). This genus was named *Opisthoporus* by Manter (1947) but the name was preoccupied and Manter (1949) replaced it with *Postporus*. Cable and Hunninen suppressed *Anallocreadium* Simer, 1929 as identical with *Homalometron* Stafford, 1904, a decision that was corroborated by Manter (1947) who added a new species, *Homalometron elongatum*, from *Gerres cinereus* taken in Florida. The description of the new species was supplemented by Bravo-Hollis and Manter (1957). G. C. Miller (1959) redescribed *Homalometron armatum* (MacCallum, 1895) and discussed the status of the genus; *Homalometron pearsei* (Hunter and Bangham, 1932) was suppressed as a synonym of *H. armatum*. The subfamily name was emended to Homalometroninae by Manter (1963a, p. 105).

A significant contribution to the life-cycle and taxonomy of the Homalometroninae was made by Hopkins (1937) who described and figured the cercariae of *Anallocreadium armatum* and *Microcreadium parvum*, two fresh-water species from the snail, *Amnicola peracuta*, taken in the Little Brazos River, Brazos County, Texas. The cercariae encysted in *Musculium ferrissi* and the older metacercariae resembled the adults so closely that tentative identification was possible. Hopkins stated (p. 94), "Both cercariae have: (1) cuticular spines, (2) setae or "sensitive hairs" covering the sides of the body, (3) three pairs of setae on the tail, (4) pigmented eyespots, (5) a long slender tail, and (6) no stylet. The second characteristic distinguishes them from the trichocercous cercariae of *Lepocreadium* and *Pharyngora*, the fifth distinguishes them from the microcercous or cotylocercous cercariae of *Plagioporous*, *Hamacreadium* and *Sphaerostoma*, and they differ from the ophthalmoxiphidiocercariae of *Allocreadium* and *Crepidostomum* in the first, second, third, and fifth characteristics. This new type of cercaria, which may be called the Anallocreadine type, may be defined as follows: Distome cercariae with straight slender tail, undivided sac-like excretory bladder, pigmented eyespots, cuticular spines, no stylet, numerous setae on sides of body, and three pairs of setae on tail; developing in rediae in freshwater snails."

---

[2] The name Lepocreadiidae was first published in the Zoological Record for 1934, but the parts were issued separately and before the volume. Whether Part VI, which contains the literature on the Trematoda, was issued in 1934 or 1935 is uncertain.

MATERIAL AND METHODS

Anallocreadine cercariae were found emerging from *Hydrobia minuta* collected in the areas of Boothbay Harbor, Maine, and Woods Hole, Massachusetts. During the past six years, hundreds of *H. minuta* from the New England coast have been examined by isolation and crushing, and from 2% to 5% of the snails yielded the anallocreadine cercaria. Other trematode infections in *H. minuta* include an unidentified heterophyid, two unidentified microphallid species, and three species whose life-cycles have been worked out: *Levinsenilla minuta* Price, 1934 by Stunkard (1958); *Notocotylus minutus* n. sp., by Stunkard (1960a); and *Himasthla compacta* n. sp., by Stunkard (1960b).

The anallocreadine cercariae from *H. minuta* are very similar to those from *Amnicola peracuta* described by Sewell (1937) as larvae of *Anallocreadium armatum* [= *Homalometron armatum* (MacCallum, 1895) Manter, 1947]. These cercariae are produced in rediae in *H. minuta* and emerge principally at night. They are photonegative and accumulate on the dark side of a finger-bowl. They swim tail first, with the body contracted and bent ventrally until it appears almost spherical, and the tail extended and lashing vigorously. This action of the tail lifts the larva in the water and pulls it along. The cercariae creep by alternate attachment of the oral and ventral suckers, and under a cover-glass the body, which is almost cylindrical, often rolls over as it moves. The acetabulum is protrusible and in lateral view may appear almost stalked. Alive and stained with neutral red or Nile blue sulphate, the internal structures, other than the excretory tubules and flame-cells, are often conspicuous.

These cercariae were left in finger-bowls with various invertebrates from the area where the snails were collected. They penetrated and encysted in *Gemma gemma,* uninfected *H. minuta,* and small polychaete annelids. Attempts to infect various crustaceans and small fishes gave only negative results. Laboratory-infected *G. gemma* were fed to *Fundulus heteroclitus* that had been held in the laboratory for three to four weeks and very small, recently excysted, worms were recovered, identical with those of natural infections. Moreover, a series of specimens, from juveniles to mature, gravid *H. pallidum* were taken from natural infections of *F. heteroclitus.* The youngest worms were recently excysted metacercariae and the gravid worms each contained a few, 3 to 15, large, unembryonated eggs. Some of these eggs were incubated in sea water and later fed to laboratory-raised *H. minuta.* Six weeks later, these snails were liberating cercariae, identical with those of natural infections. The production of experimental infections in both snails and fishes establishes the life-cycle of the parasite. All stages were studied alive and after fixation and staining. Serial sections of infected snails gave details of the asexual generations, but sporocysts were not observed.

STAGES IN THE LIFE-CYCLE

Adult

Sexually mature worms have been described by Linton (1901; fig. 354); Stafford (1904); Manter (1926; figs. 54, 55, 56); and M. J. Miller (1941; fig. 2) who restudied the specimens in the Stafford collection. Linton's figure of the type specimen is reproduced (Fig. 1). Manter (1931) listed *Distomum globiporum*

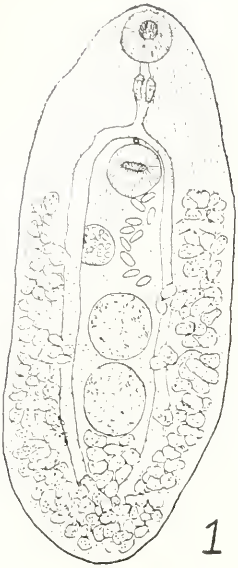
Rudolphi of Linton, 1905 from *Fundulus majalis* and *Leistomus xanthurus* and the worms designated as (*Lepocreadium*) *serospinosum* sp. inq. of Nicoll, 1909, as synonyms of *Homalometron pallidum*. As additional hosts, Linton (1940) listed *Menticirrhus saxatilis, Morone americana, Pseudopleuronectes americanus, Tautoga onitis* and *Bairdiella chrysura*. It is probable that specimens from some of these hosts have not been identified correctly, and there is the further possibility that worms taken from the intestine of predatory fishes may have been ingested with prey. In the present study, the largest specimen without eggs measures 1.12 mm. in length and is shown in Figure 3. Ovigerous worms measure from 0.80 to 2.80 mm. in length. The eggs are few, 3 to 15; 0.10 to 0.11 mm. in length and 0.06 to 0.07 mm. in breadth. They are not embryonated when passed.

Metacercaria

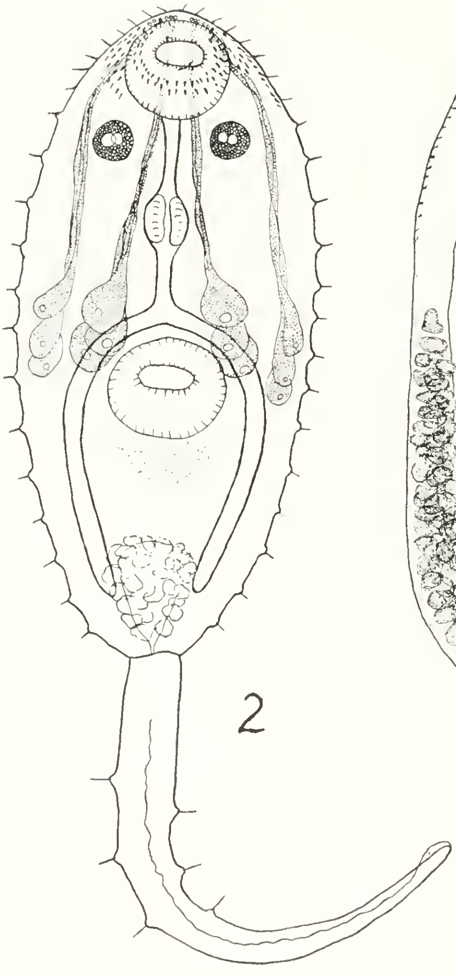
On encystment, the worms are bent ventrally so that the ends tend to approach each other and the suckers may appear almost opposite one another. The ocelli usually are situated near one end of the cyst (Fig. 6). The cysts are oval, 0.08 to 0.10 mm. in length and 0.075 to 0.808 mm. in width. The wall is very thin and excystment is easily accomplished. A recently encysted worm, released and pressed to study the excretory system and then fixed, is 0.24 mm. long and 0.11 mm. wide; the oral sucker is 0.048 mm. in diameter and the acetabulum, much flattened, is 0.056 mm. in diameter. A much older specimen of natural infection, released and measured without pressure, was 0.26 to 0.30 mm. long and 0.13 to 0.20 mm. wide; the oral sucker was 0.057 to 0.064 mm., the acetabulum 0.062 to 0.064 mm., and the pharynx 0.024 to 0.026 mm. in diameter. In this specimen the pigment of the eye-spots was becoming diffuse and scattered. These measurements show that there is little development or growth in the second intermediate host.

Redia

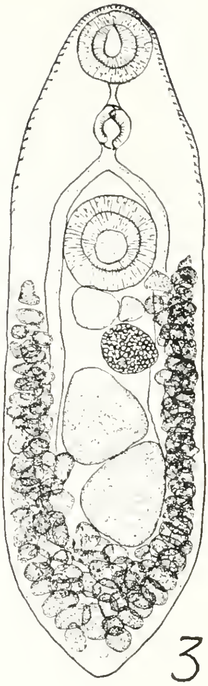
In this species, as in many digenetic trematodes, each redia produces one or more daughter rediae before the formation of cercarial embryos. The rediae are oval, to pyriform, to elongate, with a pointed posterior end. The body wall is composed of circular, longitudinal and oblique muscles, and although there are no foot-like protrusions, the smaller rediae are very mobile. As they fill with cercariae, the ability to change shape is restricted. Figure 5 shows a mother and daughter redia of experimental infection. The mother is 0.240 mm. long, 0.176 mm. wide, and the pharynx is 0.052 mm. in diameter. The daughter redia is 0.126 mm. long, 0.032 mm. wide and the pharynx is 0.026 mm. in diameter. The cercarial embryo is 0.148 mm. long and 0.074 mm. wide. Figure 4 shows a daughter redia of average size. It is 0.375 mm. long, 0.125 mm. wide, and the pharynx is 0.055 mm. in diameter. Larger rediae measure up to 0.72 mm. in length, 0.22 mm. in width and with pharynges from 0.057 to 0.065 mm. in diameter. The pharynx is protrusible and retractile; when retracted there is a distinct oral funnel. In small rediae the intestine extends one-half or more of the length of the body, but it does not increase as the redia enlarges and is more and more restricted to the anterior end of the body. It contains yellow droplets and amorphous blackish material, the residue of ingested snail tissue. The excretory system is double; there are



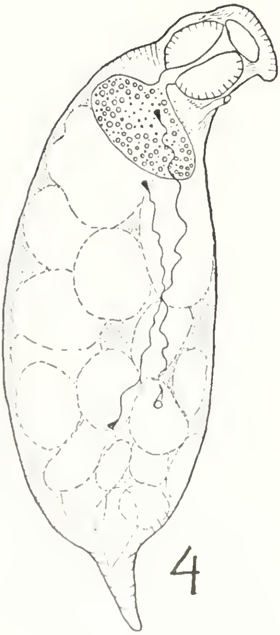
1



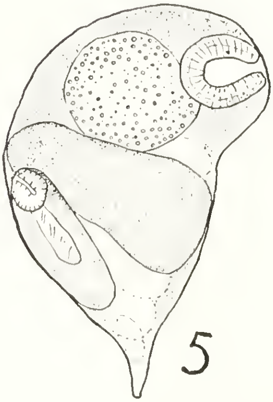
2



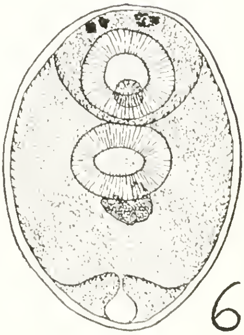
3



4



5



6

excretory pores on either side of the body, about one-third of the body length from the posterior end. From each pore, a tubule extends forward past the middle of the body where it divides; a recurrent branch passes backward past the excretory pore and terminates in a flame-cell; the anterior branch soon divides and each branch ends in a flame-cell as shown in Figure 4. The birth-pore is ventral, at the level of the posterior edge of the pharynx, and when the redia is pressed its contents may be extruded through the pore. The snail is very small, the cercariae are relatively large, and are produced in small numbers.

Cercaria

The cercariae were studied alive and after fixation and staining. Live study is far more rewarding, especially when vital dyes, neutral red and Nile blue sulphate, are employed. Stained lightly with neutral red, the granules in the cells that form the wall of the excretory bladder stain a brick red; the contents of the intestine form spherules of deep dark red; the penetration glands stain a lighter red and the cells of the germinal complex take a light lilac color. The body is oval to elongate; it measures 0.14 to 0.325 mm. in length and 0.08 to 0.135 mm. in width. Under pressure of a cover glass the apparent size of the larva and its organs increases and the body may measure 0.50 by 0.18 mm. The tail is slightly subterminal; retracted, it is much shorter than the body and since body and tail are ordinarily not extended at the same time, when the tail is extended it is longer than the body. It varies from 0.095 to 0.230 mm. in length and the tip is papilliform. The body has lateral papillae, each of which bears a seta, but the setae are easily lost. The tail has three pairs of lateral papillae with setae 0.008 to 0.009 mm. long. They are situated near the middle of the tail; also there is a low dorsoventral finfold, that is somewhat wider distally. The acetabulum is slightly posterior to the middle of the body, the same size as the oral sucker, and measures 0.05 to 0.06 mm. in diameter. The cuticula is spined, the spination more conspicuous anteriorly and diminishing posteriorly, where in young cercariae it can be seen only with oil immersion magnification. There is a pair of ocelli, equipped with lenses, situated directly above the transverse commissure of the nervous system and anterior to the pharynx. There are twelve to fifteen granules, each about one micron in diameter in any diameter of the ocellus; the ocelli measure 0.016 to 0.020 mm. in diameter. The mouth is subterminal, the oral sucker is 0.050 to 0.060 mm. in diameter. The prepharynx is longer than the esophagus and the pharynx, 0.02 to 0.024 mm. in diameter, is immediately posterior to the level of the ocelli, with the

---

EXPLANATION OF PLATE

All figures are of *H. pallidum*

FIGURE 1. Type specimen, after Linton, 1901; ventral view.

FIGURE 2. Cercaria, from *Hydrobia minuta,* drawn from sketches made during study of living specimens.

FIGURE 3. Largest non-gravid specimen from intestine of *Fundulus heteroclitus,* natural infection; dorsal view.

FIGURE 4. Daughter redia, from natural infection of *H. minuta.*

FIGURE 5. Mother redia, with daughter and first cercarial larva; experimental infection of *H. minuta.*

FIGURE 6. Encysted metacercaria, from *Gemma gemma;* experimental infection.

anterior edge of the pharynx at the level of the posterior edge of the ocelli. The digestive tract bifurcates a short distance anterior to the acetabulum. The ceca are long, ending blindly at the sides of the excretory vesicle. There are six pairs of penetration glands on each side of the body, situated anterior and lateral to the acetabulum. The ducts of three gland cells pass forward on the median side of the ocellus and the ducts of the other three glands pass lateral to the ocellus. All open at the anterior end of the body, above the mouth. The excretory vesicle is saccate, its wall formed by a large number of glandular cells which stain intensely with vital dyes. From the anterolateral sides of the vesicle, collecting ducts pass forward to the level of the acetabulum where they divide into anterior and posterior branches. Each branch bears three groups of flame-cells and although the pattern has not been completely worked out, it appears to be $(3 + 3 + 3) + (3 + 3 + 3)$.

Systematic Relations

The morphology of the sexually mature worms and of the successive larval stages of *H. pallidum* supports the proposal of Cable and Hunninen (1942) that the genus *Homalometron* is a member of the family Lepocreadiidae. Despite the accrual of much information on the morphology, life-cycles, larval stages and bionomics of the lepocreadiid trematodes, there is much difference of opinion concerning their systematic relations. The subfamilies Lepocreadiinae and Homalometrinae were included by Yamaguti (1958) in the family Allocreadiidae. The family as conceived contained 25 subfamilies and includes such diverse and disparate types that inherent unity is lacking. Indeed, in a footnote the author predicated (p. 95), "This family name is used tentatively in a broad sense."

On the basis of life-history studies, Cable and Hunninen (1942) removed *Deropristis* from the family Acanthocolpidae and included it in the Lepocreadiidae. Subsequent authors, Dawes (1946), Skrjabin (1954), Bykhovskii and Dubinina (1954), and Yamaguti (1958) retained *Deropristis* in the Acanthocolpidae but Skrjabin (1958) removed the Deropristinae from the Acanthocolpidae and elevated the subfamily, which contained also *Skrjabinopsolus* Ivanov and Murygin, 1937 (syn. *Pristotrema* Cable, 1952) and *Pristicola* Cable, 1952, to family rank. Peters (1961) accepted the family Deropristiidae which he emended to include two subfamilies: Deropristiinae Cable and Hunninen, 1942 and Cestrahelminae n. subfam., erected to contain *Cestrahelmins* Fischthal, 1957, described originally as a genus of "uncertain familial relationships." The action of Peters was approved by Fischthal and Kuntz (1963) but Baer and Joyeux (1961) retained the Deropristinae as a subfamily in the Lepocreadiidae.

Present concepts concerning the limits and composition of the Lepocreadiidae derive in large part from the studies of Cable and Manter. In a series of papers, Manter has reported on the morphology, distribution-patterns, zoogeographical affinities and taxonomy of the trematode parasites of marine and fresh-water fishes. He (1933) erected a new genus, *Eurypera*, to contain *Distomum gyrinus* Linton, 1907 and other species, and a new family Euryperidae. Since the generic name was preoccupied, Manter (1934) renamed the genus, *Megapera*, and the family Megaperidae. Cable (1954) worked out the life-cycle of *M. gyrina* and noted the similarity of its cercaria to the trichocercous larvae of the Lepocreadiidae. He postulated close relationship between the Megaperidae and Lepocreadiidae. Manter

(1963a) compared morphological features of the two families and stated (p. 99), "Megaperidae Manter, 1934 is reduced to a subfamily, Megaperinae, of the Lepocreadiidae." He observed (p. 105), "The derivation of the generic name *Homalometron* Stafford, 1904, requires Homalometroninae rather than "Homalometrinae." Dollfus (1950) erected a new genus, *Trematobrien,* and a new family, Trematobrienidae, to contain a species, *Trematobrien haplochromios,* from *Haplochromis moffati* of the Belgian Congo. Manter (1962) noted the resemblance of *Trematobrien* and *Crassicutis* Manter, 1936 and borrowed the type specimens of *T. haplochromios.* Study of this material led to the suppression of Trematobrienidae and the inclusion of *Trematobrien* in the subfamily Homalometroninae of the Lepocreadiidae.

In a survey of the family, Manter (1963b) stated that the lepocreadiids are almost entirely parasites of marine fishes, and that the subfamily Homalometroninae is of particular significance since its members occur in marine, brackish, and a few fresh-water fishes. He discussed the geographical dispersal of the subfamily and the possible significance of present distribution for the phylogeny and evolution of the hosts and their parasites. Relating hostal and geographical relations, Manter observed that *Crassicutis* Manter, 1936 occurs in cichlid fishes of Yucatan and the related genus, *Trematobrien* Dollfus, 1950 is in cichlids of the Congo. These are tropical fresh-water fishes of South and Central America and the West Indies and of Africa, Syria, Madagascar and South India. Many can live in brackish water, which suggests a coastal connection between the continents involved. *Crassicutis* has two marine species in the Gulf of Mexico and two species in Lake Valencia, Venezuela. The genus, *Microcreadium* Simer, 1929, is in the fresh-water drum, *Aplodinotus,* which belongs to a predominantly marine family. North of the tropics, the subfamily is represented by the genus *Homalometron* whose species occur in marine, brackish and a few fresh-water fishes. Manter concluded (p. 59), "In general, the subfamily illustrates an evolution from marine ancestors, through secondary-division hosts, to become parasites of freshwater fishes." The idea of host-specificity and parallel evolution in parasitic flatworms is not new. It was discused by Stunkard (1957) who maintained (p. 261), "Present information indicates that the adoption of the parasitic habit occurred at an extremely remote period and that the evolution of parasitic life-cycles, with accompanying adaptations of parasites, has proceeded hand in hand with the evolution of their hosts. The presence of closely related trematodes in marine and freshwater hosts may be explained on the assumption that the primitive hosts harbored the ancestors of present species and that the hosts separated and differentiated into marine and fresh-water types."

Summary

The successive stages in the life-history of *Homalometron pallidum,* a parasite of *Fundulus heteroclitus,* described by Linton, 1901 and named by Stafford, 1904, have been discovered and identified. The asexual generations are in *Hydrobia minuta* and the metacercarial stages are encysted in *Gemma gemma, H. minuta,* and small polychaete annelids which serve as secondary intermediate and transfer hosts. Knowledge of larval as well as adult stages gives a better basis for determination of the evolution and systematics of the allocreadiid-leprocreadiid trematodes. The subfamily Homalometroninae contains marine, brackish-, and fresh-

water species. Manter (1963) discussed the geographical dispersal of the group and the possible significance of present distribution for the phylogeny and evolution of the parasites and of their hosts. In his opinion, the subfamily illustrates an evolution from marine ancestors, through secondary-division hosts, to become parasites of fresh-water fishes. Results of the present investigation support his hypothesis.

LITERATURE CITED

Baer, J. G., and C. Joyeux, 1961. Classe des Trématodes. *In:* P. P. Grassé, Traité de Zoologie, Tome IV. Masson et Cie., Paris.

Bravo-Hollis, Margarita, and H. W. Manter, 1957. Trematodes of marine fishes of Mexican waters. X. Thirteen Digenea, including nine new species and two new genera, from the Pacific coast. *Proc. Helm. Soc., Washington,* **24**: 35–48.

Bykhovskii, B. E., and M. N. Dubinina, 1954. Data on the systematics of digenetic trematodes of the family Acanthocolpidae Lühe, 1909. (In Russian.) *Zool. Zhur.*, **33**: 788–793.

Cable, R. M., 1954. Studies on marine digenetic trematodes of Puerto Rico. The life cycle in the family Megaperidae. *J. Parasitol.*, **40**: 202–208.

Cable, R. M., and A. V. Hunninen, 1942. Studies on *Deropristis inflata* (Molin); its life history and affinities to trematodes of the family Acanthocolpidae. *Biol. Bull.*, **82**: 292–312.

Dawes, B., 1946. The Trematoda. Cambridge University Press.

Dollfus, R. P., 1950. Trématodes récoltés au Congo belge par le Professeur Paul Brien. *Ann. Mus. Belg. Congo, C.-Dierk., R. 5,* **1**: 136 pp.

Fischthal, J. W., and R. E. Kuntz, 1963. Trematode parasites of fishes from Egypt. Part V. Annotated record of some previously described forms. *J. Parasitol.*, **49**: 91–98.

Hopkins, S. H., 1937. A new type of allocreadid cercaria: the cercaria of *Anallocreadium* and *Microcreadium*. *J. Parasitol.*, **23**: 94–97.

Hunter, G. W., III, and R. V. Bangham, 1932. Studies on fish parasites of Lake Erie. I. New trematodes (Allocreadiidae). *Trans. Amer. Micros. Soc.*, **51**: 137–152.

Linton, E., 1901. Parasites of fishes of the Woods Hole region. *Bull. U. S. Fish Comm. for 1899:* pp. 405–492.

Linton, E., 1940. Trematodes from fishes mainly from the Woods Hole region, Massachusetts. *Proc. U. S. Nat. Mus.*, **88** (3078): 1–172.

Looss, A., 1894. Distomen unserer Fische und Frösche. Neue Untersuchungen über Bau und Entwicklung des Distomenkörpers. *Biblioth. Zool.*, Heft **16**: 296 pp.

Looss, A., 1899. Weitere Beiträge zur Kenntniss der Trematoden-Fauna Aegyptens, zugleich Versuch einer natürlichen Gleiderung des Genus *Distomum* Retzius. *Zool. Jahrb., Syst.*, 12: 521–784.

Looss, A., 1900. Nachträgliche Bemerkungen zu den Namen der von mir vorgeschlagenen Distomidengattungen. *Zool. Anz.,* **23**: 601–608.

Looss, A., 1902. Ueber neue und bekannte Trematoden aus Seeschildkröten. Nebst Erörterung zur Systematik und Nomenclatur. *Zool. Jahrb., Syst.*, **16**: 411–894.

Looss, A., 1907. Zur Kenntnis der Distomenfamilie Hemiuridae. *Zool. Anz.*, **31**: 585–620.

MacCallum, W. G., 1895. On the anatomy of two distome parasites of freshwater fish. *Vet. Mag.*, 2 (7): 401–412.

Manter, H. W., 1926. Some North American fish trematodes. *Illinois Biol. Monogr.,* **10** (2): 138 pp.

Manter, H. W., 1931. Some digenetic trematodes of marine fishes of Beaufort, North Carolina. *Parasitol.*, **23**: 396–411.

Manter, H. W., 1933. A new family of trematodes from marine fishes. *Trans. Amer. Micros. Soc.*, **52**: 233–242.

Manter, H. W., 1934. *Megapera*, a new name for the trematode genus *Eurypera* Manter. *Trans. Amer. Micros. Soc.*, **53**: 293.

Manter, H. W., 1936. Some trematodes of cenote fish from Yucatan. Publ. Carnegie Instit. Wash. (457): 33–38.

MANTER, H. W., 1941. Two unusual trematodes. *J. Parasitol.* **27** (Suppl.): 26.

MANTER, H. W., 1947. The digenetic trematodes of marine fishes of Tortugas, Florida. *Amer. Midl. Natur.*, **38**: 251–416.

MANTER, H. W., 1949. An additional trematode from Tortugas, Florida, and a new name for *Opisthoporus* Manter, 1947, preoccupied. *Amer. Midl. Natur.*, **41**: 432–435.

MANTER, H. W., 1962. Notes on the taxonomy of certain digenetic trematodes of South American freshwater fishes. *Proc. Helm. Soc. Washington*, **29**: 97–102.

MANTER, H. W., 1963a. Studies on digenetic trematodes of fishes of Fiji. II. Families Lepocreadiidae, Opistholebetidae, and Opecoelidae. *J. Parasitol.*, **49**: 99–113.

MANTER, H. W., 1963b. The zoogeographical affinities of trematodes of South American freshwater fishes. *Syst. Zool.*, **12**: 45–70.

MILLER, G. C., 1959. Studies on the genus *Homalometron* Stafford, 1904 (Trematoda: Lepocreadidae), with a redescription of *H. armatum* (MacCallum, 1895). *J. Parasitol.*, **45**: 539–542.

MILLER, M. J., 1941. A critical study of Stafford's report on "Trematodes of Canadian fishes" based on his trematode collection. *Canad. J. Res., Sect. D*, **19**: 28–52.

PEARSE, A. S., 1924. The parasites of lake fishes. *Trans. Wisconsin Acad. Sci., Arts, Lett.*, **21**: 161–194.

PETERS, L. E., 1961. The genus *Skrjabinopsolus* (Trematoda: Digenea) with reference to the allocreadioid problem. *Amer. Midl. Natur.*, **65**: 436–445.

SIMER, P. H., 1929. Fish trematodes from the lower Tallahatchie River. *Amer. Midl. Natur.*, **11**: 563–588.

SKRJABIN, K. I., 1954. Trematodes of animals and man, IX (in Russian.) Akad. Nauk, SSSR. Moscow.

SKRJABIN, K. I., 1958. Trematodes of animals and man, XV (in Russian). Akad. Nauk, SSSR. Moscow.

STAFFORD, J., 1904. Trematodes from Canadian fishes. *Zool. Anz.*, **27**: 481–495.

STOSSICH, M., 1903. Note distomologiche. *Boll. Soc. adriat. di sc. nat. Trieste*, **21**: 193–201.

STUNKARD, H. W., 1957. Host-specificity and parallel evolution of parasitic flatworms. *Zeit. Tropenmed. u. Parasitol.*, **8**: 254–263.

STUNKARD, H. W., 1958. The morphology and life-history of *Levinseniella minuta* (Trematoda: Microphallidae). *J. Parasitol.*, **44**: 225–230.

STUNKARD, H. W., 1960a. Further studies on the trematode genus *Himasthla* with descriptions of *H. mcintoshi* n. sp., *H. piscicola* n. sp., and stages in the life-cycle of *H. compacta* n. sp. *Biol. Bull.*, **119**: 529–549.

STUNKARD, H. W., 1960b. Studies on the morphology of *Notocotylus minutus* n. sp., a digenetic trematode from ducks. *J. Parasitol.*, **46**: 803–809.

WALLIN, I. E., 1909. A new species of the trematode genus *Allocreadium* with a revision of the genus and a key to the subfamily Allocreadiinae. *Trans. Amer. Micros. Soc.*, **29**: 50–64.

YAMAGUTI, S., 1958. Systema Helminthum. Vol. I. The digenetic trematodes. Interscience Publ., Inc., New York.

Vol. 126, No. 2 April, 1964

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

CHEMICALLY AND PHOTOPERIODICALLY INDUCED DIAPAUSE DEVELOPMENT IN THE EUROPEAN CORN BORER, OSTRINIA NUBILALIS[1]

STANLEY D. BECK AND NANCY ALEXANDER

Department of Entomology, University of Wisconsin, Madison 6, Wisconsin

The term "diapause development" was coined by Andrewartha (1952) to designate the physiological changes requisite to the termination of the diapause state and the resumption of morphogenesis. The physiological processes constituting diapause development have never been positively identified.

The principal schemes that have been advanced to explain diapause and diapause development can be classified into two categories: (1) developmental inhibitor theories, and (2) biochemical defect theories. Under the first category are those explanations in which the insect's normal developmental patterns are postulated to have been temporarily suppressed by an accumulation of an inhibiting metabolite (Roubaud, 1922), a "diapause factor X" (Bodine, 1932), "diapause factors X and Y" (Salt, 1947), or a growth-inhibiting "diapause hormone" (Schneider, 1950; Hinton, 1953; Fukaya and Mitsuhashi, 1957; de Wilde and de Boer, 1961). Such hypotheses postulated that the insect's growth remained in an arrested state until the inhibitor had been eliminated. Diapause development would, therefore, be identified as the process of biochemical degradation or elimination of the growth inhibitor. This process was usually assumed to be accomplished more rapidly at low temperatures than at high temperatures, thus accounting for the frequent observation that diapause termination requires prolonged exposure to low temperatures (0° to 10° C.). The "developmental inhibitor" theories of diapause have proved to be of limited value, because they have generally proved to be inconsistent with the results of detailed experimental analysis (see Lees, 1955, for review).

"Biochemical defect" theories of diapause postulate that the normal morphogenic sequence is blocked, during diapause, by the absence of an essential substance —a metabolite, enzyme, or hormone. The currently accepted theory is of this type, and treats diapause as a growth hormone deficiency syndrome. This concept of

---

[1] Approved for publication by the director of the Wisconsin Agricultural Experiment Station. This study was supported in part by a research grant (GM-07557) from the National Institutes of Health.

Copyright © 1964, by the Marine Biological Laboratory

diapause was developed largely through research on the pupal diapause of *Hyalophora cecropia* and other large saturniids (Williams, 1946, 1947, 1948, 1952). Diapause is thought to be caused by a failure (for unknown reasons) of the neurosecretory cells of the brain to provide the substances required for brain hormone production. The absence of brain hormone results in inactive prothoracic glands and, consequently, no production of ecdysone. Without the latter hormone, growth and differentiation are arrested and metabolism is suppressed. By this theory, diapause development has been characterized as a renewal of brain activity (for unknown reasons) and the subsequent reestablishment of normal hormone production (Van der Kloot, 1955; Williams, 1956).

Some aspects of the "hormone deficiency" theory of diapause have been confirmed in other instances of pupal diapause (Highnam, 1958; Schoonhoven, 1962; Ichikawa *et al.*, 1956), larval diapause (Church, 1955; Fukaya and Mitsuhashi, 1957), and adult diapause (de Wilde and de Boer, 1961). It is not at all certain, however, that all instances of diapause involve a shut-down of brain neurosecretion as the specific limiting biochemical defect (Van der Kloot, 1960; de Wilde, 1961; Cloutier *et al.*, 1962); in some cases other components of the endocrine system may be defective.

The present study is a product of a long-term research program on the physiological nature of diapause development and the role of periodism in the control of growth phenomena. The experimental insect employed, *Ostrinia nubilalis,* displays a facultative larval diapause that is induced primarily by short-day photoperiods (about 12-hour scotophases) (Beck and Hanec, 1960; Beck, 1962). The temperature range required for diapause development is nearly identical to that required for morphogenesis (McLeod and Beck, 1963). Diapause development is strongly influenced by photoperiod, and diapause can be reinforced in mature larvae through an appropriate manipulation of photoperiod (McLeod and Beck, 1963). These characteristics make the species an excellent experimental form for detailed study of diapause development.

Materials and Methods

The methods for rearing the European corn borer and for inducing diapause by means of short-day photoperiods (12 hours of light and 12 hours of dark per day) have been presented in detail in previous papers (Beck, 1962; McLeod and Beck, 1963), and need not be reiterated here.

Borer larvae in diapause were removed from the rearing medium at 22 days of age. They were then placed individually in shell vials, each of which contained a strip of moistened absorbent paper. The larvae were then used experimentally.

In studying the effect of photoperiod on diapause development, a technique was employed that was based on the experimental results of McLeod and Beck (1963). This method involved exposure of the diapause larvae to long-day photoperiods (16 hours of light and 8 hours of dark per day) for a period of 10 days. This treatment resulted in near-completion of diapause development. Larvae so treated were then placed under experimental conditions (short-day or continuous dark) for an additional 10 days, following which they were returned to long-day conditions and observed for pupation. Pupation is a post-diapause event, and occurs well after the completion of diapause development. In the absence of a well-defined criterion marking the completion of diapause development, pupation was taken as the measured endpoint.

Chemical treatments were by injection into the abdominal hemocoel, employing a 30-gauge hypodermic needle mounted on a microinjector. A standard volume of 5 microliters was used in all cases. The chemicals administered were dissolved in water at concentrations calculated to provide the desired dosage in 5 microliters.

RESULTS AND DISCUSSION

Photoperiod and diapause development. Diapause borer larvae, 22 days of age, were subjected to different photoperiodic schedules and were then observed for pupation. The results (Table I) demonstrate some of the effects of photoperiod on the rate of diapause development. Of those larvae that were subjected to long-day conditions (Table I, Schedule A), 50% had pupated by the 31st day. This developmental rate was not significantly changed by transferring the larvae to continuous darkness after 20 days of long-day exposure (Schedule B). The developmental rate established during the 20 days of long-day photoperiods was apparently unaffected by the dark treatment. The results from Schedules C and D also show about the same developmental rates, indicating that the rate was established during the first 10 days of treatment and was unaltered by subsequent periods of continuous darkness.

TABLE I

Effect of different photoperiodic treatments on diapause development in the European corn borer (all larvae were reared under a short-day photoperiod and were 22 days of age at the beginning of the experiment)

Schedule	Photoperiodic treatment		Post-treatment photoperiod	Days to 50% pupation*	
	1st 10 days	2nd 10 days		Avg.	Range
A	LD**	LD	LD	31	29–34
B	LD	LD	DD***	27	25–32
C	LD	DD	LD	29	26–34
D	LD	DD	DD	30	26–34
E	LD	SD†	LD	42	37–44
F	LD	SD	DD	>50	—
G	LD	SD	SD	>50	—

* Number of days from beginning of experiment until 50% of the experimental group had pupated. The range values are the extremes from experimental replicates.

** LD = Long day (16-hour photophase, 8-hour scotophase).

*** DD = Continuous darkness.

† SD = Short day (12-hour photophase, 12-hour scotophase).

Under Schedule E of Table I, borers were exposed to long-day photoperiods for 10 days, were returned to short-day conditions for 10 days, and were then held under long-day conditions for pupation. These larvae reached the 50% pupation point at 42 days, a delay of about 10 days in comparison to the pupation rate among larvae on the previously discussed schedules. The 10-day delay in diapause development corresponded to the 10 days that the larvae spent under a short-day photoperiod. This response shows two points of interest: (1) exposure to short-day photoperiods during the second 10 days of the treatment sharply reduced the rate of diapause development, and (2) diapause development is accumulative, because the effect of the first 10 days under long-day photoperiods was not reversed or lost as a result of the later short-day treatment. If, however, the short-day treatment was followed by holding conditions of continuous darkness (Schedule F), or continued short days (Schedule G), the low rate of diapause development was maintained. Very few of the larvae (from 10% to 25%) under Schedules F and G had pupated by the end of the experimental time of 50 days. The low developmental rate established in response to short-day photoperiods was maintained under conditions of continuous darkness just as it was under continued exposure to short-day photoperiods. The role of photoperiods in diapause development appears to be that of determining the developmental rate.

The results from schedules B, C and D, contrasted with those from Schedule F, suggest that continuous darkness is photoperiodically neutral; that is, it is a *status quo* condition under which the developmental rate established by previous photoperiods is maintained. Since it has been previously shown (Beck and Apple, 1961) that exposure to continuous light promotes a rapid diapause development, it is apparent that continuous darkness and continuous light exert quite different effects on diapause development.

Only a small percentage (25% to 34%) of diapausing corn borer larvae that were held indefinitely from 22 days of age under either darkness or short-day photoperiods eventually pupated. Experimental measurements of diapause developmental rates under long-day and short-day conditions have indicated that one day of continuous light or long-day photoperiod (16 hours of light per day) resulted in an increment of diapause development equivalent to that requiring approximately 5 days of a short-day photoperiod (12 hours of light per day).

The above results are interpreted as showing that diapause in the European corn borer is not a state of *arrested* development. It is, instead, a physiological state in which the rate of developmental processes has been much reduced; the low developmental rate results in a greatly prolonged fifth larval instar (the diapause stadium) and in low oxygen consumption and other biochemical characteristics that have been found to be associated with the diapause state. Although we have dealt only with the European corn borer, it seems likely that diapause in other species may similarly involve varying degrees of developmental rate reduction rather than outright arrest; if so, the term "diapause development," as proposed by Andrewartha, reflects a legitimate concept, and is not "paradoxical," as labeled by Harvey (1962).

Other workers have also found some photoperiodic effects on form determination and diapause induction to be accumulative, with long-day effects not being completely reversed by subsequent short-day photoperiods (de Wilde, 1958; Beck and Hanec, 1960; Müller, 1962; Norris, 1962; Adkisson *et al.*, 1963; Barker *et al.*, 1963; Lees, 1963). Obviously the characteristic is general, and not peculiar to either the European corn borer or the phenomenon of diapause development.

Chemical treatment and diapause development. A study was made to determine whether or not diapause development could be accelerated by the administration of different chemicals. It was hoped that some clues to the identity of biochemical processes involved in diapause development might thus be obtained.

Water balance has frequently been implicated in insect diapause and neurosecretion (Slifer, 1946; Koidsumi, 1952; Bucklin, 1953; Nayar, 1960), and the water content of diapausing corn borer larvae has been shown to be lower than that of nondiapause larvae (Beck and Hanec, 1960). The European corn borer is also known to require contact moisture before postdiapause morphogenesis can occur (Babcock, 1924). Diapausing borer larvae were treated with different amounts of water at different ages and by several different means of administration, including injection into the hemocoel, direct introduction into the foregut and hindgut, and direct introduction into the tracheal system. In no case was diapause development accelerated by water, although it was found that the larvae could tolerate about 7 microliters of distilled water injected into the hemocoel.

A number of pharmaceutical agents, known to be central nervous system stimulants of mammals, were tested at several concentrations. It was hoped that one or more of the stimulants would promote brain hormone production (or release), thereby accelerating diapause development. The substances administered to 22-day-old diapause borers included amphetamine phosphate, methyl phenidate hydrochloride, pipradrol hydrochloride, imipramine hydrochloride, pentylenetetrazol, nethamine, and ephedrine sulfate. Water solutions were injected, and the treated larvae were held under either continuous darkness or a long-day photoperiod. All results were negative; no acceleration of diapause development was observed.

Hogan (1961, 1962) reported that urea and certain other ammonium compounds would terminate the embryonic diapause of a cricket, *Acheta commodus* (Walk.). This finding was tested with the European corn borer by injecting groups of diapausing larvae with different amounts of ammonium acetate. The treated larvae were then held in continuous darkness and observed for pupation. Ammonium acetate had a pronounced stimulating effect on the rate of diapause development (Table II). The greatest response (% pupating) was obtained with a dosage of from 600 to 800 μg. per larva. This was a massive dosage, as mature

TABLE II

Effect of different amounts of ammonium acetate on the rate of diapause development in the European corn borer. (40 larvae per treatment; all treatments were by injection in 5 μl. water)

Ammonium acetate dosage (μg.)	Response after 45 days in continuous darkness		
	Mortality (%)	Survival	
		Pupae (%)	Larvae (%)
0 (control)	15	0	85
200	40	0	60
300	80	8	12
400	40	20	40
500	58	28	15
600	58	40	2
800	58	40	2
1000	63	32	5
5000	100	0	0

TABLE III

Effects of different ammonium compounds on the rate of diapause development in the European corn borer. (All treatments were by injection in 5 μl. water; 40 larvae per treatment)

Compound	Dosage (μg.)	Response after 45 days in continuous dark		
		Mortality (%)	Survival	
			Pupae (%)	Larvae (%)
Ammonium acetate	600	58	40	2
Ammonium carbonate	500	70	0	30
	600	43	30	27
	800	80	20	0
Urea	50	20	0	80
	100	60	20	20
	500	80	10	10
	800	50	0	50
Ammonium oxalate	100	40	10	50
	300	100	0	0
Ammonium tartrate	100	70	0	30
	500	85	0	15
	800	100	0	0

borer larvae average only 100 mg. in body weight. That the response was to the ammonium ion rather than to the anion was demonstrated by the effects of several other ammonium compounds (Table III). These results demonstrate an important point of similarity between larval diapause of the European corn borer and embryonic diapause in a cricket.

Although a number of the ammonium compounds stimulated diapause development and pupation, most were rather toxic at the high dosage levels required. In general, ammonium acetate was the least toxic and most efficient of the substances tested, and it was adopted as the standard for further research. Attempts were made to improve the efficiency of the system for administering ammonium acetate to the larvae. One method tried was to use an ammonium acetate solution to moisten the paper strips in the vials in which the larvae were held; no response was obtained. Ammonium acetate solutions were introduced into the lumen of the hindgut by the use of small plastic tubes. This route of administration was effective, but the operation was difficult and inexact because of leakage. Effective amounts of the chemical could also be introduced into the foregut, but again offered no operational advantages over injection into the hemocoel. The results of these experiments demonstrated only that the borer digestive system was capable of absorbing ammonium acetate while in diapause. In an effort to avoid high postoperative mortality, serial injections of small amounts over a period of several days were tried. The mortality and response data showed no significant improvement over the administration of a single massive dose. The addition of different amounts of ammonium acetate to the larval rearing medium did not reduce the incidence of diapause among borer larvae reared under a short-day photoperiod.

A possible explanation for the ammonium effect on diapause development is that ammonium ions are required for the synthesis of other nitrogenous compounds, and a massive influx of ammonium forces the synthetic process to an effective nondiapause rate of production. According to this hypothesis, administration of the biochemical substrates actually involved in the synthesis would be effective at greatly reduced concentrations. In pursuing this hypothesis, we have tested a large number of amino and other nitrogenous compounds, but with uniformly negative results. Carbamyl phosphate, tryptamine, 5-hydroxytryptamine, several amino acids, urea cycle intermediates, RNA synthesis intermediates, proline cycle intermediates, and several others have been tested. This aspect of the problem is under current investigation, but for the present we know the response only as the "ammonium ion effect."

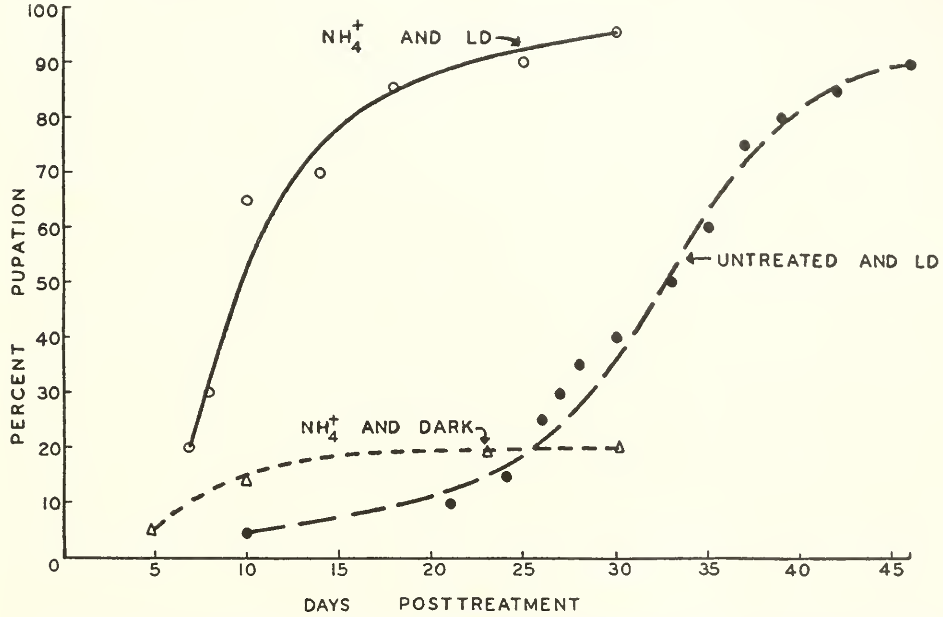


FIGURE 1. The combined effects of ammonium acetate (400 μg./insect) and photoperiod on diapause development in the European corn borer. (Untreated dark control record not plotted, because none pupated.)

Combined ammonium and photoperiodic effects. Ammonium ions accelerate diapause development, and long-day photoperiods exert the same effect. There may, therefore, be an interaction between the two accelerating factors. This hypothesis was tested in a series of experiments in which suboptimal doses of ammonium acetate were administered to diapause larvae, which were then held for pupation under either a long-day photoperiod or continuous darkness. The pupation records are shown in Figure 1. Diapause borers treated with 400 μg. of ammonium acetate pupated at a high rate if held under a long-day photoperiod (50% pupating within 10 days), but at a low rate if held in the dark (only 20% within 30 days). Untreated control larvae reached the 50% pupation point in 32 days when under a long-day schedule, but none of the untreated dark controls pupated during the 46 days of the experiment.

When held under a long-day photoperiod, groups of larvae treated with 200 μg. of ammonium acetate per insect pupated at a rate no greater than that observed among borers not treated with ammonium ions. An intermediate response was obtained when larvae were treated with 300 μg. of ammonium acetate per insect.

Ammonium ions greatly accelerated diapause development, under post-treatment conditions of either darkness or a long-dark photoperiod. Ammonium ions did not eliminate the influence of photoperiod on diapause development, however. The factors that determine the rate difference in diapause development established by different photoperiodic schedules continued to operate in the presence of large quantities of ammonium acetate. On the basis of this evidence, it is suggested that ammonium ions are involved in biochemical processes constituting diapause development, but that some step in the utilization of ammonium ions is rate-limited through the action of photoperiod.

Summary and Conclusions

1. Diapause development in European corn borer larvae is rate-controlled by photoperiod. The rate of diapause development under conditions of continuous light or long-day photoperiods is approximately 5 times that occurring under a short-day photoperiod.

2. The rate of diapause development under conditions of continuous darkness depends upon the rate established by the photoperiods to which the larvae were exposed before being placed in the dark. Diapause larvae transferred from a short-day photoperiod into darkness continue to develop at the short-day rate. Conversely, diapause larvae exposed to about 10 days of long-day photoperiod continue to undergo diapause development at the long-day rate when transferred to dark conditions.

3. Although the rate of diapause development may be changed by changes in the photoperiod, diapause development is not reversible. Diapause development summates during the period of diapause.

4. Attempts to accelerate diapause development experimentally through increasing the water content of the larvae, or by the administration of chemical nervous system stimulants were uniformly unsuccessful.

5. Diapause development was experimentally accelerated by the administration of massive doses of ammonium acetate or other ammonium compounds. This finding indicates a probable similarity between larval and embryonic diapause development.

6. The ammonium ion effect did not obviate the role of photoperiod. The rate of diapause development was strongly influenced by photoperiod among larvae that had been treated with ammonium ions. The rate of diapause development observed in the presence of both ammonium ions and long-day photoperiods greatly surpassed the rates observed among larvae exposed to either factor alone.

LITERATURE CITED

Adkisson, P. L., R. A. Bell and S. G. Wellso, 1963. Environmental factors controlling the induction of diapause in the pink bollworm, *Pectinophora gossypiella* (Saunders). *J. Ins. Physiol.*, **9**: 299–310.

Andrewartha, H. G., 1952. Diapause in relation to the ecology of insects. *Biol. Rev.*, **27**: 50–107.

Babcock, K. W., 1924. Environmental studies on the European corn borer *Pyrausta nubilalis* Hubn. *J. Econ. Ent.*, **17**: 120–125.

Barker, R. J., A. Mayer and C. F. Cohen, 1963. Photoperiod effects in *Pieris rapae*. *Ann. Ent. Soc. Amer.*, **56**: 292–294.

Beck, S. D., 1962. Photoperiodic induction of diapause in an insect. *Biol. Bull.*, **122**: 1–12.

Beck, S. D., 1963. Physiology and ecology of photoperiodism. *Bull. Ent. Soc. Amer.*, **9**: 8–16.

Beck, S. D., and J. W. Apple, 1961. Effects of temperature and photoperiod on voltinism of geographical populations of the European corn borer, *Pyrausta nubilalis* (Hbn.). *J. Econ. Ent.*, **54**: 550–558.

Beck, S. D., and W. Hanec, 1960. Diapause in the European corn borer *Pyrausta nubilalis* (Hbn.). *J. Ins. Physiol.*, **4**: 304–318.

Bodine, J. H., 1932. Hibernation and diapause in certain Orthoptera. III. Diapause—a theory of its mechanism. *Physiol. Zoöl.*, **5**: 549–554.

Bucklin, D. H., 1953. Termination of diapause in grasshopper embryos cultured *in vitro*. *Anat. Rec.*, **117**: 535.

Church, N. S., 1955. Hormones and the termination and reinduction of diapause in *Cephus cinctus* Nort. *Canad. J. Zool.*, **33**: 339–369.

Cloutier, E. J., S. D. Beck, D. G. R. McLeod and D. L. Silhacek, 1962. Neural transplants and insect diapause. *Nature*, **195**: 1222–1224.

Fukaya, M., and K. J. Nitsuhashi, 1957. The hormonal control of larval diapause in the rice stem borer, *Chilo suppressalis*. I. Some factors in the head maintaining larval diapause. *Jap. J. Appl. Ent. & Zool.*, **1**: 145–154.

Harvey, W. R., 1962. Metabolic aspects of insect diapause. *Ann. Rev. Ent.*, **7**: 57–84.

Highnam, K. C., 1958. Activity of the brain/corpora cardiaca system during pupal diapause 'break' in *Mimas tiliae* (Lepidoptera). *Quart. J. Micr. Sci.*, **99**: 73–88.

Hinton, H. E., 1953. The initiation, maintenance, and rupture of diapause: a new theory. *Entom.*, **86**: 279.

Hogan, T. W., 1961. The action of urea on diapause in eggs of *Acheta commodus* (Walk.). *Austral. J. Biol. Sci.*, **14**: 419–426.

Hogan, T. W., 1962. The effect of ammonia on the rate of termination of diapause in eggs of *Acheta commodus* (Walk.). *Austral. J. Biol. Sci.*, **15**: 538–542.

Ichikawa, M., J. Nishiitsutsuji-Uwo and K. Yashika, 1956. Studies on the insect metamorphosis. VI. Effect of low temperature on the morphogenesis of *Luchdorfia* pupae. *Mem. Coll. Sci., Univ. Kyoto, Ser. B*, **23**: 19–26.

Koidsumi, K., 1952. Water content and the hormone center for the pupation of hibernating larvae of *Chilo simplex* Butler. *Annot. Zool. Jap.*, **25**: 156–172.

Lees, A. D., 1955. The Physiology of Diapause in Arthropods. Cambridge University Press.

Lees, A. D., 1963. The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton. III. Further properties of the maternal switching mechanism in apterous aphids. *J. Ins. Physiol.*, **9**: 153–164.

McLeod, D. G. R., and S. D. Beck, 1963. Photoperiodic termination of diapause in an insect. *Biol. Bull.*, **124**: 84–96.

Müller, H. J., 1962. Über den Saisondimorphen Entwicklungszyklus und die Aufhebung der Diapause bei *Aleurochiton complanatus* (Baeren-sprung). *Ent. Exp. & Appl.*, **5**: 124–138.

Nayar, K. K., 1960. Studies on the neurosecretory system of *Iphita limbata* Stal. VI. Structural changes in the neurosecretory cells induced by water content. *Zeitschr. f. Zellfor.*, **51**: 320–324.

Norris, M. J., 1962. Diapause induced by photoperiod in a tropical locust, *Nomadacris septemfasciata* (Serv.). *Ann. Appl. Biol.*, **50**: 600–603.

Roubaud, E., 1922. Sommeil d'hiver cedant a l'hiver chez les larves et nymphes de Muscides. *C. R. Acad. Sci.*, **174**: 964–968.

Salt, R. W., 1947. Some effects of temperature on the production and elimination of diapause in the wheat stem sawfly. *Canad. J. Res., Section D, Zool. Sci.*, **25**: 66–68.

Schneider, F., 1950. Die Entwicklung des Syrphidenparasiten *Diplazon fissorius* Grav. *Mitt. Schweiz. Ent. Ges.*, **23**: 155–194.

Schoonhoven, L. M., 1962. Diapause and the physiology of host-parasite synchronization in *Bupallus piniarus* L. (Geometridae) and *Eucarcelia rutilla* Vill. (Tachinidae). *Arch. Néerland Zool.*, **15**: 111–174.

Slifer, E. H., 1946. The effect of xylol and other solvents on diapause in grasshopper eggs; together with a possible explanation for the action of these agents. *J. Exp. Zool.*, **102**: 333–356.

Van der Kloot, W. G., 1955. The control of neurosecretion and diapause by physiological changes in the brain of the cecropia silkworm. *Biol. Bull.*, **109**: 276–294.

Van der Kloot, W. G., 1960. Neurosecretion in insects. *Ann. Rev. Ent.*, **5**: 35–52.

de Wilde, J., 1958. Perception of the photoperiod by the Colorado potato beetle (*Leptinotarsa decemlineata* Say). *Proc. Xth Inter. Congr. Ent.*, **2**: 213–218.

de Wilde, J., 1961. Extrinsic control of endocrine functions in insects. *Bull. Res. Council Israel,* **10B**: 36–52.

de Wilde, J., and J. A. de Boer, 1961. Physiology of diapause in the adult Colorado beetle. II. Diapause as a case of pseudo-allatectomy. *J. Ins. Physiol.*, **6**: 152–161.

Williams, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

Williams, C. M., 1947. Physiology of insect diapause. II. Interaction between pupal brain and prothoracic glands. *Biol. Bull.*, **93**: 89–98.

Williams, C. M., 1948. Physiology of insect diapause. III. The prothoracic glands in the cecropia silkworm with special reference to their significance in embryonic and post-embryonic development. *Biol. Bull.*, **94**: 60–65.

Williams, C. M., 1952. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. *Biol. Bull.*, **103**: 120–138.

Williams, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the cecropia silkworm. *Biol. Bull.*, **110**: 210–218.

PROCTODONE, AN INSECT DEVELOPMENTAL HORMONE[1]

STANLEY D. BECK AND NANCY ALEXANDER

Department of Entomology, University of Wisconsin, Madison 6, Wisconsin

This paper presents the results of a study of the endocrinology of growth processes associated with the diapause and postdiapause states of the lepidopteran, *Ostrinia nubilalis.* Insect growth and diapause have been studied intensively by many workers, and there is a voluminous body of extant literature on the subject. In view of the profusion of recent reviews (Wigglesworth, 1959; Van der Kloot, 1960, 1961; Gilbert and Schneiderman, 1961; Harvey, 1962), there is no need for a detailed consideration of published literature here, except as necessary to define the conceptual framework within which the study was undertaken.

According to currently accepted concepts of insect growth, the first step in the endocrinological sequence is the production of brain hormone by neurosecretory cells. Brain hormone activates the prothoracic glands, which then secrete the growth and molting hormone, ecdysone. Diapause is generally considered to be caused, primarily, by a failure of the neurosecretory cells to produce brain hormone (or its precursors), although nothing is known of the cause of such failure. The absence of brain hormone results in an arrest of growth and differentiation, and in a suppression of both biosynthetic and bioenergetic metabolism (Williams, 1946; Harvey, 1962). The concept that diapause is caused only by an absence of brain hormone is so widely accepted that some workers have extirpated the brains from insects not in diapause and subsequently used the brainless insects as experimental animals in a state of diapause (Ichikawa and Nishiitsutsuji-Uwo, 1960; Ichikawa and Takahashi, 1959; Kobayashi and Nakasone, 1960).

During the period of diapause, progressive physiological changes occur that eventually lead to resumption of active growth; these unidentified changes constitute *diapause development* (Andrewartha, 1952). The end-point of diapause development is currently considered to be the renewal of brain activity and the reestablishment of normal hormone production (Van der Kloot, 1955; Williams, 1956).

The present study has involved a reconsideration of the physiological nature of diapause and diapause development, and also a re-evaluation of the position of brain hormone in the sequence of developmental events. One of the underlying factors prompting this study was the accumulating evidence of instances of diapause that appear to involve a suppression of endocrine functions other than brain hormone production alone (Fukaya and Mitsuhashi, 1958; Van der Kloot, 1960; de Wilde, 1961; de Wilde and de Boer, 1961; Cloutier *et al.*, 1962; Beck, 1963).

---

[1] Approved for publication by the director of the Wisconsin Agricultural Experiment Station. This study was supported in part by a research grant (GM-07557) from the National Institutes of Health.

Another reason underlying our reinvestigation of the identity and sequence of endocrine functions in insect growth phenomena was the inadequacy of the current theory to account satisfactorily for several aspects of insect photoperiodism—diapause induction, diapause development, and seasonal dimorphism. This point may be illustrated, at least in part, by the experimental results obtained in this laboratory in studies on the photoperiodism of the European corn borer, *Ostrinia nubilalis.* Facultative diapause is induced in this species by short-day photoperiods, and occurs in the mature fifth instar larva. Growth rates during the early larval stages are not reduced by rearing the larvae under diapause-inducing short-day photoperiodic schedules, although nearly all of the larvae enter diapause upon attaining larval maturity. This means that the brain-prothoracic gland-corpora allata endocrine system functioned normally through four complete molting cycles, despite the influence of the photoperiod. Unless one makes some complicated assumptions concerning the effect of photoperiod on changing developmental thresholds, it is difficult (by current concepts) to rationalize the delay of the overt photoperiodic response—diapause—until the fifth instar is attained.

This picture is further complicated by the finding that long-day photoperiods experienced early in larval life modify the intensity of diapause in borers subsequently transferred to short-day photoperiods for the completion of larval growth (Beck and Hanec, 1960). Similarly, long-day photoperiods experienced during early diapause exert an eventual effect on the completion of diapause development (Beck and Alexander, 1964). Such hold-over accumulative effects of long-day photoperiods have been reported not only in the European corn borer, but also in the Colorado potato beetle (*Leptinotarsa decemlineata*) (de Wilde, 1958), the pink bollworm (*Pectinophora gossypiella*) (Adkisson *et al.*, 1963), and in the dimorphism of the vetch aphid (*Magoura viciae*) (Lees, 1963). These several effects, plus the finding of Cloutier *et al.* (1962) that the brain of the corn borer is at least potentially active from an endocrinological standpoint during diapause, suggest that the neurosecretory system of the insect brain may be under the control of a second, unidentified physiological system that is responsive to photoperiod (Beck, 1963).

There is some evidence that the sites of photoperiodic reception in insects include the brain itself (Lees, 1960; Shakhbazov, 1961; Harker, 1960). There is also evidence, however, that the photoperiodic sites of reception are not exclusively cephalic (de Wilde, 1958). The unidentified system suggested to influence neurosecretory processes should be sensitive to photoperiod, but need not be either a cephalic or a central nervous system component. If photoperiodic stimuli influence two interacting physiological systems, photoperiodic responses such as diapause and dimorphism might result from the effects of the photoperiodic regime on the phase relationships between them, provided they are rhythmic growth-controlling functions. One of the rhythmic functions would probably be that of neurosecretion. The other would be the postulated process to which neurosecretion is closely linked.

By this admittedly speculative view, diapause would be the result of the physiological rhythms' being forced into an out-of-phase relationship through the phase-setting effects of the photoperiod. Much of the study reported below was designed to test this "phase hypothesis" of insect developmental phenomena.

METHODS AND MATERIALS

The methods used for rearing European corn borer larvae and for inducing either diapause or uninterrupted development were described in an earlier paper (Beck, 1962). The photoperiodic and chemical treatments utilized to accelerate diapause development have also been described in detail (McLeod and Beck, 1963; Beck and Alexander, 1964). Many of the experiments reported herein involved the use of ligation and tissue implant techniques. In general, the procedures used were similar to those employed by many other workers, and warrant no detailed description. In so far as practicable, aseptic surgical techniques were employed.

In most of the experiments, the measured end-point of diapause development was taken as pupation. It should, however, be understood that pupation is actually a postdiapause event. The occurrence of pupation depends not only upon the completion of diapause development, but also upon the completion of a postdiapause molting cycle. Larvae that had been ligated or severely injured by surgical manipulations were frequently unable to undergo ecdysis to the pupal stage. The formation of pupal cuticle was taken as the primary criterion of pupation in all such cases.

RESULTS

A group of diapausing European corn borer larvae, 22 days of age, was placed in a lighted incubator at 30° C. and a photoperiodic schedule of 16 hours of light and 8 hours of darkness per day (long-day photoperiod). These larvae began to pupate about 20 days later; by 30 days half of them had pupated, and all had attained the pupal stage by the forty-fifth day. Comparable larvae placed at 30° C. under a short-day photoperiod (12 hours of light and 12 hours of darkness per day) did not begin to pupate until after the sixtieth day, and at the time the last larva died, 122 days after the beginning of the experiment, only 33% of the group had attained pupal status. Such experiments have clearly demonstrated that diapause development was rate-controlled through the influence of the environmental photoperiod. Very pronounced acceleration of development was obtained by injecting 400 micrograms of ammonium acetate into each larva before placing it into the long-day incubator. Diapausing larvae so treated began to pupate on the eighth day; 50% had pupated by the twelfth day, and all had pupated prior to the thirtieth day (Beck and Alexander, 1964).

Gross determination of the locations of sites of photoreception was made by placing diapausing borers in partially masked glass tubes and exposing them to different photoperiods. The inside diameter of the tubes used was too small to allow the larvae to turn around, and both the inside and outside surfaces of the tube were masked with black paint. The larvae were oriented within the tubes in such a manner that only a selected part of the insect was under the unpainted portion of the tube. Two portions of the larvae were exposed to the influence of photoperiods: (1) the head only or (2) the abdomen only (abdominal segments 7 to 10). Controls consisted of groups of larvae placed in unpainted tubes, but otherwise treated in the same way as the experimental larvae.

Two types of experiments were run: (1) re-induction of the short-day rate of development in larvae that had been previously exposed to long-day photoperiods

TABLE I

Effect of abdominal ligations on the photoperiodic response of diapausing European corn borer larvae (long-day photoperiod)

Position of ligature	Larvae treated (no.)	Incidence of pupation* (%)
None (control)	40	90
7th abdominal segment	110	10
8th abdominal segment	60	35
9th abdominal segment	30	67

* Per cent pupating within 40 days post-treatment.

for 10 days; and (2) long-day acceleration of diapause development in larvae not previously exposed to long-day photoperiods. The experimental results showed that exposure of either the head or the abdomen to the incident photoperiod produced a partial response, as compared to the response of the control group, in which both head and abdomen were exposed. The responses were considered partial in the sense that the rate of diapause development adjusted to the photoperiod less rapidly among the partly masked larvae than among the controls. This effect was interpreted as an indication that extra-cephalic photoreception occurs in the corn borer, with the receptor system probably being located in the abdomen. Our results are in good agreement with those of de Wilde (1958), who found extra-cephalic photoperiodic sensitivity in adult Colorado potato beetles.

The possible existence of a photoperiodically sensitive abdominal system was investigated through a series of ligation and surgical experiments. Diapause development was effectively prevented in larvae that had been bisected by tightly applied ligatures (Table I). Ligations were made sufficiently tight to insure destruction of nervous as well as circulatory communication between the divided portions of the insect (surgical removal of the parts posterior to the ligature did not alter the responses obtained). Ligations posterior to the seventh abdominal segment were progressively less effective. Diapause development is apparently

TABLE II

Determination of the critical period for abdominal system participation in diapause development in European corn borer larvae (long-day photoperiod and ammonium acetate treatment on day 0)

Post-treatment (days)	Post-operation survival (no.)	Pupation within 20 days (%)
0	37	3
1	33	21
2	24	21
3	15	53
4	16	50
5	30	30*

* Pupation incidence from the fifth day on was low because the molting cycle was well advanced in many of the larvae, and abdominal ligations inflicted severe mechanical injury from which the larvae were unable to recover completely.

TABLE III

Time table for diapause development and pupation in the European corn borer (long-day photoperiod and 400 μg. ammonium acetate per larva)

Post-treatment (days)	Abdominal system	Brain system	Prothoracic glands
0	+*	+	+
1	+	+	+
2	±	+	+
3	−	+	+
4	−	+	+
5	−	±	+
6	−	−	±
7	−	−	−
8 ------	---------- pupation begins ----------		-------

* + = required; − = not required.

dependent upon functions occurring in the abdomen, principally the seventh and eighth abdominal segments. Since diapause development is thought to lead to the activation of the known endocrine organs, all of which are located in the head and prothorax, the inhibition of diapause development by seventh abdominal segment ligation indicated that the effect of photoperiod must, in part, be exerted *via* an unidentified abdominal system.

The time relationships involved in the participation of the abdominal system in diapause development were determined (Table II). In these experiments, borer larvae in diapause were treated with 400 μg. of ammonium acetate and transferred to a long-day photoperiod. At daily intervals, groups of larvae were ligated at the seventh abdominal segment and then returned to the long-day incubator. The larvae were then observed for pupation occurring within 20 days from the beginning of the experiment. Under these conditions, the per cent pupating rose sharply among larvae ligated after the second day. From the third day on, posterior ligation did not prevent pupation. The "critical period" for abdominal system participation in diapause development was, therefore, between two and three days under these experimental conditions. When tested under long-day photoperiods but without ammonium ions, the critical period was found to be about 12 days. Treatment with ammonium acetate tended to reduce individual variation within the experimental groups, and therefore permitted greater precision in the determination of the developmental sequence.

Ligations at the seventh abdominal segment, made during the first 5 days of the experiment discussed above (Table II), resulted in pupation of only the portion of the larvae anterior to the ligature. However, larvae that were ligated on the sixth and later days developed pupal cuticle posterior as well as anterior to the ligature. This response showed that the critical period for ecdysone-dependence of the pupation process under these experimental conditions was approximately 6 days.

Removal of the heads of diapause larvae periodically after ammonium acetate injection and the beginning of long-day exposure demonstrated that the critical period for brain hormone production was about 5 days. The developmental schedule during diapause development and postdiapause morphogenesis to the pupal stage was concluded to be approximately as shown in Table III.

Role of central nervous system in diapause development

The possibility that the photoperiodic effect on diapause development is mediated by the central nervous system was investigated. Other workers (Lees, 1960; Shakhbazov, 1961) have reported that the neurosecretory cells of the brain are directly sensitive to light stimuli, and Prosser (1934) and Kennedy (1958, 1963) have reported that the sixth abdominal ganglion of the ventral nerve cord in some arthropods is sensitive to photostimuli. These considerations, especially in view of the fact that the terminal ventral ganglion of the borer larva lies in the seventh abdominal segment, led us to test the hypothesis that the neurosecretory cells of the larval brain are subject to secretory control by impulses originating in the terminal ventral ganglion. The experimental data discussed above are inconsistent with the concept that the neurosecretory activity of the brain is suppressed during diapause by inhibiting impulses from the posterior ganglion. If such were the case, ligation anterior to the ganglion should have cut off such impulses and released the brain from the inhibited state. Obviously, such was not the case; seventh-segment ligations prevented, rather than promoted, diapause development. The experimental evidence lends better support to the hypothesis that brain-stimulating impulses might be involved in diapause development.

TABLE IV

Effects of ventral nerve cord severance on diapause development

Post-operative photoperiod	Larvae used (no.)	Pupation (%)
Long-day	43	35
Short-day	15	7
Dark	15	0

If diapause developmental changes in the neurosecretory system were induced by impulses from the terminal ganglion, severing of the ventral nerve cord immediately anterior to that ganglion should prevent diapause development. This hypothesis was tested experimentally by severing the ventral nerve cords of diapause borer larvae at the sixth abdominal segment. The operation was performed by microcautery, and the operated larvae were held under three different photoperiodic conditions: long-day, short-day, and continuous dark. The post-operative mortality was exceedingly high (65%), but the pupation data (Table IV) clearly showed that cutting the ventral nerve cord did not prevent diapause development, and the photoperiodic response was still manifested. Dissection of each of the pupae obtained confirmed that the nerve cord had been severed in every case. On the basis of these data, it was concluded that diapause development in the European corn borer does not depend on neural communication between the posterior ventral ganglion and the brain.

Earlier work in this laboratory (Cloutier *et al.*, 1962) has shown that the brain of a diapause corn borer implanted into another diapause borer results in the pupation of the recipient. Such an effect of supposedly incompetent brains was postulated to be the result of surgical alteration of membrane permeability controlling the exchange of nutrient and metabolites between the brain and the blood. In those experiments, the postoperative holding conditions were always short-day photoperiods, because it was thought that such diapause-inducing photoperiods would constitute the most stringent experimental conditions available. In the present study, the "brain barrier" effect was subjected to further experimentation. We were particularly interested in determining whether or not the effects of implanted brains were subject to photoperiodic influence.

Brains (supraesophageal ganglia only) of diapause corn borer larvae were implanted into other diapause larvae of the same age and developmental history. The operated larvae were then held under long-day, short-day, or continuous dark conditions. The effect of photoperiod on diapause development was clearly manifested (Table V, section A). A much higher percentage pupation was observed under long-day conditions than in the dark, and the short-day photoperiod produced an intermediate response. The response difference between short-day and dark was greater than expected, and will be discussed in a later section. The implanted brains were recovered by dissection after pupation, and it was found that they did not become associated with the nervous system of the host insect, so the effect of photoperiod could not have been mediated by the insect's central nervous system.

TABLE V

Photoperiod, brain activity, and diapause development in the European corn borer

Section	Implanted brain activity condition	Recipient condition	Post-treatment photoperiod	Operated larvae (no.)	Observed pupation (%)
A	Diapause	Diapause	Long-day	12	92
	Diapause	Diapause	Short-day	18	78
	Diapause	Diapause	Darkness	17	35
B	Diapause	Diapause + 7th seg. ligation	Short-day	20	0
	Diapause	Diapause + 7th seg. ligation	Dark	22	0
C	Nondiapause	Diapause	Short-day	23	100
	Nondiapause	Diapause + 7th seg. ligation	Short-day	12	67

When diapause brains were implanted into diapause larvae that had been ligated at the seventh abdominal segment, no diapause development occurred (Table V, section B). It was, therefore, apparent that the abdominal system was functional in larvae receiving implanted brains, and was required for the completion of diapause development.

When fully active, nondiapause larval brains were implanted into diapause larvae, all of the recipients pupated (Table V, section C). Ligation of the recipient larvae at the seventh abdominal segment did not prevent diapause development and pupation when a nondiapause brain was implanted. These were considered to be crucial experiments, and the results allowed us to draw two important conclusions: (1) the diapause brain is not competent, in itself, to induce morphogenesis; and

(2) the abdominal system is required only until the endocrine capability of the brain is fully established. The experimental results also demonstrate that ligation of larvae at the seventh abdominal segment does not so interfere with the mechanics and hydraulics of the molting process that it blocks pupation. However, mechanical interference was previously shown to be of importance only if the molting cycle was well advanced (Table II).

All of the above experiments on the role of the brain, ventral nerve cord, and abdominal system in a diapause development demonstrated that the abdominal system is intimately involved in photoperiodism and diapause development. It is also apparent that the influence of the abdominal system is not communicated to the brain system *via* the central nervous system. The conclusion is drawn that the brain-stimulating factor must be elaborated in the abdominal system and carried by the blood to the brain, where neuroendocrine functions are influenced. The participation of the abdominal system in the process of diapause development must, therefore, be in the form of a hormone.

Endocrine activity of the abdominal system

Two prominent organs are present in the seventh and eighth abdominal segments of the European corn borer larva. One is the terminal ganglion of the ventral nerve cord, and the other is the anterior portion of the hindgut, termed the anterior intestine. These two structures were examined for histological evidence of secretory activity.

The terminal abdominal ganglion represents a fusion of the seventh and eighth primitive abdominal ganglia, and it innervates the terminal segments, muscles, integument, and posterior digestive tract (Snodgrass, 1935). As mentioned above, the abdominal ganglia of crayfish and some other arthropods have been shown to be photosensitive, with the light-induced nerve impulses originating in the ganglion or in the peripheral nerves, rather than in specialized photoreceptors. If a comparable situation prevails in the borer larva, the photo-induced impulses would stimulate local effectors rather than impulse transmission to the brain. The body wall of the borer larva is translucent, and should constitute no serious barrier to light-stimulation of the ganglion or peripheral nerves. A large plexus of peripheral nerves leads into the terminal ganglion from the posterior extremities of the insect; light-induced impulses might originate in some of these nerves. This aspect of the problem is under current investigation.

The possibility of neurosecretory activity in the posterior ganglion was investigated. A pair of neurosecretory cells was found on each lateral aspect of the ganglion. With paraldehyde-fuchsin staining method (Cameron and Steele, 1959), one cell stained purple and the other stained green. But similar pairs of neurosecretory cells were also found on each side of the ventral ganglia lying anterior to the terminal ganglion. Because the hormone involved in diapause development was traced to the body segment of the terminal ganglion, and not to any segment anterior to that ganglion, it was concluded that these neurosecretory cells were not involved in the production of the hormone in question.

Histological examination of the hindgut during early diapause and diapause development disclosed some striking changes in form and staining properties of certain cells during diapause development. These cells are in the epithelium of a portion of the anterior intestine located a short distance posterior to the bases of the Malpighian tubes, but anterior to the heavily muscled rectal sphincter.

The epithelial cells of most of the hindgut of diapausing larvae are large, contain ovoid nuclei, and possess relatively homogeneous cytoplasm. The epithelial cells of the particular area in question show evidence of secretory activity: the nuclei are multilobate and the cytoplasm contains granular inclusions and numerous small vacuoles. The granular inclusions were found to stain with paraldehyde-fuchsin in a manner similar to neurosecretory granules. It is, of course, recognized that the paraldehyde-fuchsin technique is not a specific cytochemical identification of chemical structure.

There are three lines of evidence supporting the conclusion that these proctodaeal cells produce the abdominal system hormone required to account for the experimental results presented in an earlier section. Because of its source, the name PROCTODONE is proposed for this hormone. The lines of supporting evidence are:

(1) The cytological signs of secretory activity appear at a time long prior to the manifestation of visible changes in the integument that might be indicative of the initiation of the pupal molting cycle. During diapause the lumen of the hindgut is empty, and we have seen no evidence of secretion from the proctodaeal cells into the lumen.

(2) Diapausing larvae that have been treated with ammonium acetate and transferred into long-day conditions for rapid diapause development display a greatly intensified secretory activity in the hindgut cells. The cells present a turbulent appearance, with greatly increased cytoplasmic volume, large vacuoles, numerous granules, and widespread multilobate nuclei. It is quite apparent that proctodone production has been greatly increased under the influence of ammonium ions.

(3) Evidence of a daily secretory cycle has been found. Whether under short-day, long-day, or long-day plus ammonium ions, the cell activity appears to undergo cyclic changes that are obviously regulated by the photoperiod. Under long-day conditions, the purple-staining granular inclusions accumulate during the hours of darkness, forming large dense clumps in the cytoplasm. A few hours after onset of light, the cells were found to be nearly devoid of granular inclusions, although still displaying the other signs of secretory function.

Attempts to accelerate diapause development by the implantation of fragments of active proctodone-producing tissue have not, thus far, been successful. This failure does not prove that the cells in question have no endocrine function, because extirpation and implantation of the tissue requires separation of the cells from their nervous connection. Integrity of innervation from the posterior ventral ganglion may be important to the physiological function. Because of the large size of the proctodone-producing area of the hindgut, we have not been able to implant a very large proportion of the total active tissue. The fragments implanted may have been insufficient to produce a response.[2]

---

[2] Since the submission of this paper, we have found that aqueous extracts of active proctodeal tissue accelerate diapause development when injected into late diapause borers (see *Science,* 143: 478 (1964)).

DISCUSSION

The details of the role played by proctodone in insect development have yet to be elucidated, and the following discussion is admittedly speculative. Nevertheless, a few characteristics may be inferred from the experimental results now available.

Proctodone's primary target tissue must be the brain, presumably the neurosecretory cells. This interpretation is supported by the data presented in Table V, where it was shown that the implantation of a fully active nondiapause brain obviated the role of the proctodone-producing abdominal system. Most certainly, proctodone is not a diapause hormone, in the sense of an inhibitor of neurosecretory activity. The experimental evidence is consistent only with the concept that proctodone is a developmental hormone in the full sense of the term.

Secretion of proctodone into the hemolymph is apparently on a daily incremental basis, although its effect on the brain system is summated during diapause development. This conclusion is supported by the finding of a secretory cycle in the hindgut epithelium, and by previously published results (Beck and Alexander, 1964) showing that diapause development is not a reversible process. In the earlier study, it was found that the rate of diapause development could be changed by changing photoperiodic conditions, but the amount of diapause development already accomplished by exposure to long-day photoperiods was not lost when the insects were returned to diapause-inducing short-day conditions. The photoperiodically induced diapause development was summated until the developmental threshold was reached, at which point photoperiod and the abdominal system apparently played no further role—diapause development was completed.

Proctodone production and brain sensitivity to proctodone are postulated to be rhythmic processes, both of which are phase-set by photoperiod. The rate of diapause development is low when these two processes are held in an out-of-phase relationship by a short-day photoperiod. A long-day photoperiod resets the rhythms in a development-promoting phase relationship. The data of Table V, section A, and the results of Cloutier *et al.* (1962) can be tentatively explained on the basis of phase relationships between the brain and abdominal systems. Implanted diapause brains were probably released from photoperiodic phase-setting, and they then adopted a free-running circadian rhythm. If the period of the brain rhythm was different from 24 hours, it would be free to drift into phase with the photoperiodically set abdominal rhythm, and diapause development would be accelerated. Diapause brains implanted into diapause borers maintained in continuous darkness were found to be less effective, and this might be explained on the basis that in the dark, both rhythmic systems were free-running with nearly identical periods, and therefore they could only very slowly drift into an entraining phase relationship.

Diapause development in the European corn borer is rate-limited by the phase relationships between the brain and abdominal systems, such that diapause development may be accelerated by factors that increase the amount of proctodone produced per day (ammonium ion effect) or by photoperiodic schedules that establish favorable phase relationships (long-day effect). A combination of these factors produces extremely rapid diapause development.

It seems most probable that proctodone will be found to play an important role in many insect growth phenomena. Although our experimental work has, thus far, dealt only with the larval diapause of the European corn borer, the published results of other workers, using a variety of insect forms, indicate a number of effects that might be explained by the postulated action of proctodone and a "phase theory" of developmental control.

Williams (1946) reported that diapause pupae of *Hyalophora cecropia* required from 10 to 12 weeks at about 5° C. in order to complete diapause development. In 1956, however, he discovered that the brains of pupae chilled for only 5 weeks would induce morphogenesis when implanted into unchilled pupae. Competence upon implantation occurred much earlier than competence *in situ*. This effect is apparently identical to that reported by Cloutier *et al.* (1962) for the European corn borer, except that photoperiod rather than temperature was involved in the latter case. Since we have traced the effect to proctodone activity in the case of the corn borer, it seems most likely that proctodone is also involved in diapause development of *Hyalophora cecropia.*

Bounhiol (1943, 1945) reported on the effects of variously placed ligatures on pupation and metamorphosis of the commercial silkworm, *Bombyx mori.* He found that an abdominal ligation, performed shortly after the larva had ceased to feed, effectively prevented pupation. At a somewhat later stage, such ligatures did not prevent pupation. Ligation of the Malpighian tubes during the developmental stage at which pupation could be prevented did not prevent the formation of pupal cuticle to some degree, but did prevent adult differentiation (Bounhiol, 1945). Bounhiol interpreted his results in terms of Malpighian tube function and the possible excretion of inhibitory hormones. His results, however, appear to be inconsistent with such an interpretation, but are in accord with the results of the current study and our interpretation that an abdominally produced hormone—proctodone—is involved in insect morphogenesis.

Davey (1962), working with the American cockroach, *Periplaneta americana,* reported that an extract of corpus cardiacum stimulated muscle contractions in the hindgut. The cardiacum factor was thought to act directly upon the epithelial cells of the anterior intestine; these cells, in turn, released an indolalkylamine which stimulated the nerves controlling muscle contractions in the posterior hindgut. Some similarity between Davey's and our results is apparent in attributing hitherto undetected physiological functions to the epithelial cells of a portion of the proctodeum. It should be noted, however, that the tryptamine compounds found to be active in the cockroach phenomenon did not produce the ammonium ion effect on diapause development in the European corn borer (Beck and Alexander, 1964).

Recently, Johnson (1963) traced neurosecretory axon pathways from the brain to various parts of the insect body, using several species of aphids. A neurosecretory pathway was traced through the ventral nerve cord to the hindgut. Such results suggest the probability of a feedback regulatory relationship between hindgut and neuroendocrine activities.

There are also lines of evidence that indicate possible proctodone participation in both adult and embryonic diapause. The ammonium ion effect was discovered by Hogan (1961), working with an embryonic diapause. The probable existence of noncephalic photoreceptors that play a part in photoperiodism was pointed out by de Wilde (1958), working with an adult diapause. Adult diapause could not be accounted for on the basis of only a suppression of neurosecretory activity (de Wilde and de Boer, 1961).

Diapause does not occur among insects in which the temperature requirements for "diapause development" are similar to the temperature requirements for growth, and which are growing under environmental conditions where photoperiodic influences are either absent or are conducive to uninterrupted growth and morphogenesis. This situation might occur in populations of species displaying facultative diapause, and in species in which diapause does not ordinarily occur. In these cases, the physiological events constituting diapause development must occur concurrently with morphogenic events. Under such "nondiapause conditions," growth and morphogenesis are not interrupted by a phase separation imposed by genetic and environmental factors, such as temperature and photoperiod, and diapause does not occur. But even in such nondiapause cases, the proctodone-neuroendocrine interaction is probably involved in the programming of growth and metamorphosis as normal components of the insect's endocrine functions. These considerations have led us to avoid calling proctodone a "diapause development hormone."

Recent attempts to formulate plausible theories of form determination have emphasized the role of both environmental and humoral factors in the exchange of genetic information, gene penetrance, and gene suppression (Wigglesworth, 1959, 1961; Williams, 1961; de Wilde, 1961). The importance of the known hormones has been stressed, but one of the main weaknesses of current theories has been their inability to account for environmental effects, particularly periodism. The proctodone-producing system and its phase relationships to other endocrine functions may lead to some clarification of some aspects of the polymorphism problem.

The ability of insects to respond to environmental rhythms of photoperiod and thermoperiod in terms of activity rhythms, metabolic rhythms, form determination, and diapause points to their possession of a time-measuring system—a biological clock (Lees, 1960; Beck, 1963). From the experimental results presented in this report, we have good reason to believe that proctodone production is a rhythmic phenomenon and a photoperiodically controlled response in the European corn borer. It seems quite probable that similar relationships will be found in other insect time-measuring systems. If such proves to be the case, meaningful experimental analysis of many developmental problems should be possible.

It is of interest to note that our finding of proctodone does not lead to a contradiction of already well-established knowledge of insect endocrine functions. What is accomplished, however, is the demonstration that proctodone production precedes brain hormone production in the endocrine sequence, and also provides a connecting link between the extrinsic and intrinsic factors controlling growth.

SUMMARY

1. Diapause development in larvae of the European corn borer, *Ostrinia nubilalis*, was shown to be dependent on a physiological factor originating in the seventh and eighth abdominal segments.

2. Under conditions of an experimentally accelerated diapause development schedule (long-day photoperiod plus ammonium acetate treatment), the action of the abdominal system was required for about two days, the brain system for about

five days, and the prothoracic system for about six days, in order to complete diapause and prepupal postdiapause development.

3. The influence of the abdominal system was shown to be transmitted by the blood stream, rather than *via* the ventral nerve cord.

4. The abdominal system was postulated to elaborate and release a hormone, whose physiological action is to stimulate the neuroendocrine system of the brain.

5. Because the abdominal system hormone was found to originate in the epithelial cells of the anterior portion of the hindgut, it was named *proctodone*.

6. Proctodone production was found to be on a 24-hour rhythmic basis, with hormone release occurring during the light hours of the photoperiod.

7. Proctodone is postulated to play a role in nondiapause growth, polymorphism, periodism, and the several forms of diapause.

LITERATURE CITED

Adkisson, P. L., R. A. Bell and S. G. Wellso, 1963. Environmental factors controlling the induction of diapause in the pink bollworm, *Pectinophora gossypiella* (Saunders). *J. Ins. Physiol.*, **9**: 299–310.

Andrewartha, H. G., 1952. Diapause in relation to the ecology of insects. *Biol. Rev.*, **27**: 50–107.

Beck, S. D., 1962. Photoperiodic induction of diapause in an insect. *Biol. Bull.*, **122**: 1–12.

Beck, S. D., 1963. Physiology and ecology of photoperiodism. *Bull. Ent. Soc. Amer.*, **9**: 8–16.

Beck, S. D., and N. Alexander, 1964. Chemically and photoperiodically induced diapause development in *Ostrinia nubilalis*. *Biol. Bull.*, **126**: 000–000.

Beck, S. D., and W. Hanec, 1960. Diapause in the European corn borer, *Pyrausta nubilalis* (Hbn). *J. Ins. Physiol.*, **4**: 304–318.

Bounhiol, J. J., 1943. Nymphose (partielle) localisée, chez des vers à soie divises en trois parties par deux ligatures. *C. R. Acad. Sci.*, **217**: 203–204.

Bounhiol, J. J., 1945. La rétention, expérimentalement provoquée, dans les tubes de Malpighi entrave la nymphose des chenilles du *Bombyx mori* L. *C. R. Acad. Sci.*, **220**: 64–65.

Cameron, M. L., and J. E. Steele, 1959. Simplified aldehyde-fuchsin staining of neurosecretory cells. *Stain Tech.*, **34**: 265–266.

Cloutier, E. J., S. D. Beck, D. G. R. McLeod and D. L. Silhacek, 1962. Neural transplants and insect diapause. *Nature*, **195**: 1222–1224.

Davey, K. G., 1962. The mode of action of the corpus cardiacum on the hindgut in *Periplaneta americana*. *J. Exp. Biol.*, **39**: 319–324.

Fukaya, M., and J. Mitsuhashi, 1958. The hormonal control of larval diapause in the rice stem borer, *Chilo suppressalis*. II. The activity of the corpora allata during the diapausing period. *Jap. J. Appl. Ent. & Zool.*, **2**: 223–226.

Gilbert, L. I., and H. A. Schneiderman, 1961. Some biochemical aspects of insect metamorphosis. *Amer. Zool.*, **1**: 11–51.

Harker, J. E., 1960. Internal factors controlling the suboesophageal ganglion neurosecretory cycle in *Periplaneta americana* L. *J. Exp. Biol.*, **37**: 164–170.

Harvey, W. R., 1962. Metabolic aspects of insect diapause. *Ann. Rev. Ent.*, **7**: 57–80.

Hogan, T. W., 1961. The action of urea on diapause in eggs of *Acheta commodus* (Walk.). *Austral. J. Biol. Sci.*, **14**: 419–426.

Johnson, B., 1963. A histological study of neurosecretion in aphids. *J. Ins. Physiol.*, **9**: 727–739.

Ichikawa, M., and J. Nishiitsutsuji-Uwo, 1960. Studies on the insect metamorphosis. VII. Effect of the brain hormone to the isolated abdomen of the Eri-silkworm, *Philosamia cynthia ricini*. *Mem. Coll. Sci. Univ. Kyoto, Series B*, **27**: 9–15.

Ichikawa, M., and S. Takahashi, 1959. Study on the endocrine activity of *Bombyx*-allatum in the diapausing *Philosamia* pupa. *Mem. Coll. Sci. Univ. Kyoto, Series B*, **26**: 249–252.

KENNEDY, D., 1958. Responses from the crayfish caudal photoreceptor. *Amer. J. Ophthamol.*, **46**: 19–26.

KENNEDY, D., 1963. Physiology of photoreceptor neurons in the abdominal nerve cord of the crayfish. *J. Gen. Physiol.*, **46**: 551–572.

KOBAYASHI, M., AND S. NAKASONE, 1960. Imaginal differentiation in artificially induced diapausing brainless pupae of the silkworm, *Bombyx mori*, by treatment of carbon dioxide or oxygen. *Bull. Seri. Exp. Sta. (Tokyo)*, **16**: 100–112.

LEES, A. D., 1960. Some aspects of animal photoperiodism. *Cold Spring Harbor Symp. Quant. Biol.*, **25**: 261–268.

LEES, A. D., 1963. The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton. III. Further properties of the maternal switching mechanism in apterous aphids. *J. Ins. Physiol.*, **9**: 153–164.

McLEOD, D. G. R., AND S. D. BECK, 1963. Photoperiodic termination of diapause in an insect. *Biol. Bull.*, **124**: 84–96.

PROSSER, C. L., 1934. Action potentials in the nervous system of the crayfish. I. Responses to illumination of the eye and caudal ganglion. *J. Cell. Comp. Physiol.*, **4**: 363–377.

SHAKHBAZOV, V. G., 1961. The reaction to the length of daylight and the light receptor of the pupa of the Chinese oak silkworm, *Antheraea pernyi* G. *Dok. Akad. Nauk., SSSR.*, **140**: (AIBS) 944–946.

SNODGRASS, R. E., 1935. Principles of Insect Morphology. McGraw-Hill Publishing Co., New York.

VAN DER KLOOT, W. G., 1955. The control of neurosecretion and diapause by physiological changes in the brain of the cecropia silkworm. *Biol. Bull.*, **109**: 276–294.

VAN DER KLOOT, W. G., 1960. Neurosecretion in insects. *Ann. Rev. Ent.*, **5**: 35–52.

VAN DER KLOOT, W. G., 1961. Insect metamorphosis and its endocrine control. *Amer. Zool.*, **1**: 3–9.

WIGGLESWORTH, V. B., 1959. The Control of Growth and Form. Cornell University Press, Ithaca, New York.

WIGGLESWORTH, V. B., 1961. Polymorphism—a tentative hypothesis. *Symp. Roy. Ent. Soc. London*, **1**: 103–113.

DE WILDE, J., 1958. Perception of the photoperiod by the Colorado potato beetle (*Leptinotarsa decemlineata* Say). *Proc. Xth Int. Congr. Ent.*, **2**: 213–218.

DE WILDE, J., 1961. Extrinsic control of endocrine functions in insects. *Bull. Res. Coun. Israel*, **10B**: 36–52.

DE WILDE, J., AND J. A. DE BOER, 1961. Physiology of diapause in the adult Colorado beetle. II. Diapause as a case of pseudo-allatectomy. *J. Ins. Physiol.*, **6**: 152–161.

WILLIAMS, C. M., 1946. Physiology of insect diapause. The role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

WILLIAMS, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the cecropia silkworm. *Biol. Bull.*, **110**: 201–218.

WILLIAMS, C. M., 1961. Insect metamorphosis: An approach to the study of growth. *In:* Growth in Living Systems. Academic Press, pp. 313–320.

ALPHA AMYLASE DEVELOPMENT IN EMBRYOS OF CRASSOSTREA VIRGINICA[1]

ROBERT E. BLACK AND E. T. PENGELLEY[2]

Department of Biology and Virginia Institute of Marine Science,[3] College of William and Mary, Williamsburg, Virginia

Numerous investigations of changes in enzyme activity during development have been made during recent years. Such studies have been reviewed by Moog (1958, 1959), Løvtrup (1959) and Brachet (1960). Relatively few quantitative investigations have been made of enzymes in embryonic invertebrates; we therefore have little knowledge of enzymic changes which are temporally associated with visible morphogenetic events in these embryos.

In this paper, data on the levels of alpha amylase in several developmental stages of the oyster are presented. This enzyme is present in considerable concentration in the crystalline style, a mucoprotein rod secreted in a diverticulum of the intestine of adult pelecypods and some gastropods (Yonge, 1926; Prosser and Brown, 1961). It was therefore of interest to determine whether any change in amylase activity accompanied the development of the larval intestine. It was also of some interest to determine whether a high activity of this enzyme would be found in larvae which had not yet begun feeding, since the levels of digestive enzymes in various adult animals sometimes depend on the presence of appropriate foods (*cf.* van Weel, 1961; Prosser and Brown, 1961).

MATERIALS AND METHODS

Adult oysters were collected from the Rappahannock River and stored in trays suspended from the laboratory pier at Gloucester Point, Virginia. The salinity at the laboratory varies during the summer from approximately 18 to 22 ppm. For collecting gametes, portions of the gonads were removed from the adults and shaken briefly in pasteurized river water having a salinity of 20 ppm. The eggs were washed by settling and fertilized. Eggs from different adults were kept separate until the percentage of fertilization could be checked at the time of first cleavage. In all batches of eggs used, more than 90% of the eggs were fertilized and developed normally. The eggs were cultured at 28° C. in a rotating flask suspended in a Warburg apparatus. The concentration of larvae was kept at about 5×10^6 per liter. The culture water contained 250 units of penicillin and 250 micrograms of streptomycin per milliliter.

Before setting up each larval culture, 5 aliquots were diluted, and two secondary aliquots of 1.0 ml. were counted from each of the primary samples. At the

---

[1] Supported by a grant (GB-710) from the National Science Foundation.

[2] Present address: Division of Biological Sciences, University of California, Riverside, California.

[3] Contribution No. 154.

appropriate developmental stage three large aliquots of embryos were collected by centrifugation, washed in pasteurized river water, and homogenized at 0° C. in 2% NaCl containing 0.02 *M* phosphate buffer at pH 6.2. Homogenization was accomplished by the use of an all-glass Tenbroeck homogenizer of small capacity.

Amylase was assayed by the method of Sumner, as described by Bernfeld (1955). For each assay 0.1 ml. of appropriately diluted homogenate was mixed at zero time with 0.4 ml. of 1% potato starch (also in NaCl phosphate buffer) in a test tube of 10 ml. capacity. After incubation at 28° C. for 30 minutes the reaction was stopped by the addition of 1.0 ml. of 3,5-dinitrosalicylate reagent (1% 3,5-dinitrosalicylate in 0.4 N NaOH containing 30% Rochelle salt). The tubes were boiled for 5 minutes, and 2.0 ml. of water were added after cooling. After 5 minutes of centrifugation at about 3000 *g*, the absorbance was determined at 540 millimicrons in a Beckman DU spectrophotometer. Standards containing known amounts of maltose were treated in an identical manner in each experiment.

Enzyme assays were always run at two levels of homogenate concentration; this provided continuous assurance that reaction rates were proportional to the amount of homogenate added. In preliminary experiments the digestion reaction was found to be nearly linear for 30 minutes.

For determinations of the extent of bacterial contamination, aliquots were removed from embryo cultures, from whole antibiotic-free embryos washed at the time of enzyme assay, and from embryo homogenates. These aliquots were spread on nutritive sea-water agar plates and incubated five days at 20° C. before counting.

In one experiment larvae of 17 and 37 hours were fixed in Bouin's fluid at 70° C., embedded in paraffin, and sectioned at 5–7 microns. Sections were stained with Delafield's hematoxylin in order to determine in a gross way the degree of formation of the larval intestine at these times, which correspond to the first detected appearance and point of maximum activity of amylase.

Determinations of total protein were made in some instances on aliquots of homogenate. These were done by the method of Lowry *et al.* (1951).

RESULTS

Development of the larval intestine

At 28° C. the "trochophore" stage is reached in about 8 hours, at which time the endoderm is a cellular mass which fills most of the blastocoele. There is a slight invagination at the blastopore, representing the archenteron and presumptive mouth. At 17 hours the archenteron cavity extends about one-half the distance between the blastopore and the site of the future anus. At this time the larva is a straight-hinge veliger. Observations on whole mounts and living embryos after 17 hours indicate that the intestinal tract is a complete, straight tube by 22 hours of development at this temperature. Sections at 17 hours show that ciliation is evident on the cells that lie in the medial part of the archenteron. By 37 hours the gut is tripartite and exhibits extensive ciliation and some muscular movement. No evidence for a style sac or other diverticulum was found in embryos up to 48 hours of age. Embryos of 37 hours and older were frequently found to possess a small mass of highly refractive material, which did not stain with hematoxylin,

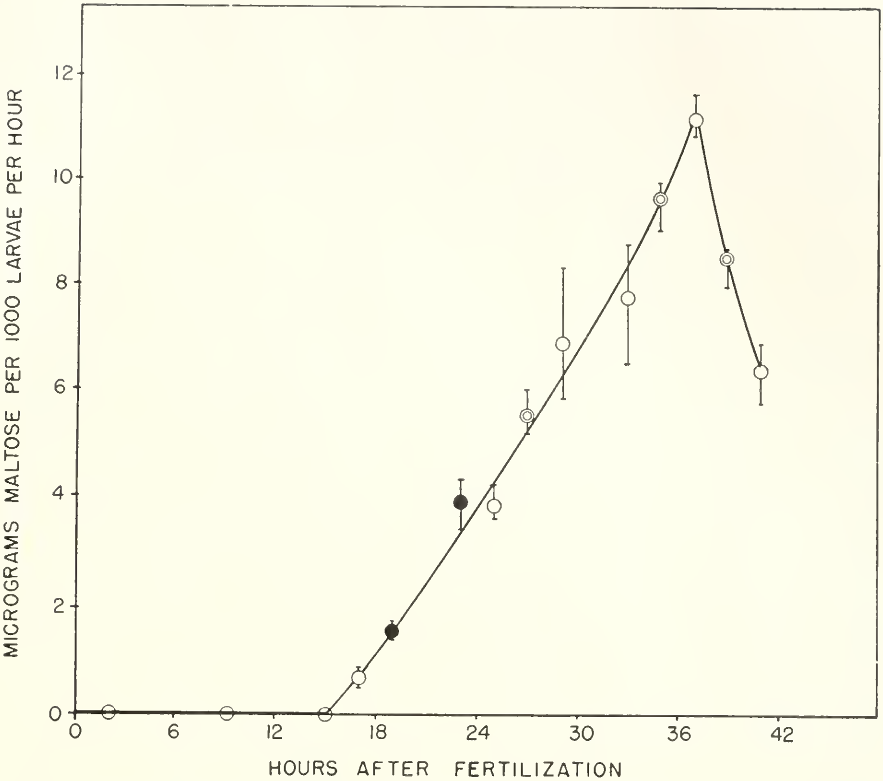


FIGURE 1. Alpha amylase activity in homogenates of developing oyster larvae, grown at 28° C. The different symbols represent different batches of eggs. The range of values obtained is shown for each stage. The incubation mixture contained 0.8% potato starch, 2% NaCl, and 0.02 *M* sodium phosphate, pH 6.2.

in the lumen of the midgut. This may represent a larval precursor to the crystalline style.

Amylase activity in homogenates

Data on enzyme activity in homogenates are presented in Figure 1. The enzyme was not detected before 17 hours of development, even with homogenate concentrations of 50% in the assay mixtures, and with incubation times up to three hours. The increase in activity between 17 and 37 hours is nearly linear. Extrapolation of this activity curve shows that the increase probably begins at about 15 hours. After 37 hours a sharp decrease in activity occurs. The decrease is evidently not a result of the release of the enzyme into the medium, since no amylase activity could be detected in water in which concentrated suspensions of 37-hour embryos had been incubated for two hours.

The average values obtained in five determinations of total protein in the homogenates are as follows ($\times 10^{-10}$ grams per egg): unfertilized eggs, 2.6 ± 0.6;

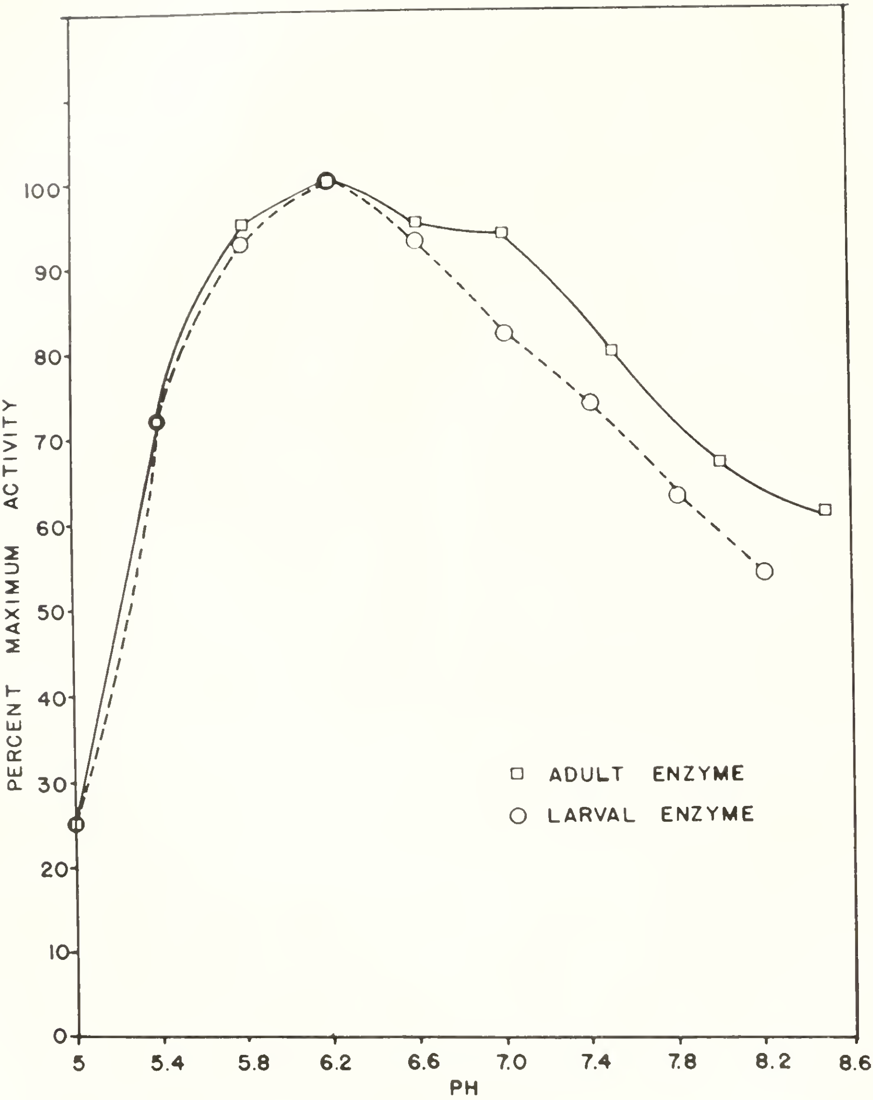


FIGURE 2. The effect of pH on the activity of amylase from 36-hour veligers and from adult crystalline styles of the oyster. All preparations contained 0.8% potato starch, 2% NaCl, 0.02 *M* sodium acetate and 0.02 *M* sodium phosphate.

20-hour veligers, 1.7 ± 0.5; 48-hour veligers, 1.8 ± 0.7. The apparent decrease in protein during the first 20 hours may be only artifactual, since it was difficult to correct for the differences in turbidity of the homogenates at the different stages. In any case the changes in enzyme activity are not reflected by any simultaneous changes in total protein.

The bacterial contamination in the cultures appeared to be low in all experiments. The plate counts varied from 0 to 0.06 bacterium per embryo when live, "antibiotic-free" embryos were plated. Counts made from the homogenates gave values of 50 to 10,000 per ml.; by comparison the homogenates contained approximately 10^6 embryos per ml. There was no correlation between the bacterial counts in homogenates and the enzyme activity in these, and it was concluded that the extent of contamination was insufficient to affect the level of enzyme activity in the assay mixtures.

Properties of larval and adult amylases

It was of interest to compare the properties of the larval enzyme with those of amylase extracted from adult styles, with respect to pH optimum, substrate specificity, and energy of activation. Crystalline styles were dissected from adults, homogenized in buffered saline solution, and centrifuged at 10,000 *g* for 5 minutes to remove insoluble, mucus-like material. Data obtained on enzyme activities in acetate-phosphate buffers of different pH are presented in Figure 2. The optimum pH for the enzyme from both sources is 6.2. A second optimum is shown by the adult enzyme at 7.0; the crystalline style may therefore contain a second amylase which is not present in the larva. Assays of enzyme activity with different substrates indicated that the enzyme from both sources digested these at the following relative rates: potato starch 1.00, "soluble" starch 0.7–0.8, and glycogen 0.3–0.5. The temperature coefficients for digestion of potato starch were found to be as follows: larval enzyme, 10–20° C., 2.29; 20–30°, 1.76; adult enzyme, 10–20°, 2.76; 20–30°, 1.85. The average energy of activation for the larval enzyme is therefore 11,845 calories per mole while that for the adult is 14,700 calories. Since neither enzyme was purified, this difference probably does not represent an actual chemical difference in the two enzymes. Both values are reasonably close to the value of 13,350 calories per mole, which has been reported for human salivary amylase (Bernfeld, 1955).

Discussion

It is apparent from Figure 1 that enzyme production or activation in the larva must decrease abruptly or stop at 37 hours. It appears possible that the decrease could result from extracellular digestion of amylase by proteolytic enzymes in the intestine; however, if enzyme production or activation were continuing at the same time, one would expect a more gradual decline in activity. It would be of interest to determine whether enzyme activity could be maintained at a high level under conditions in which amino acids and substrates are fed to the larvae. It is conceivable that the decrease in enzyme activity is a result of exhaustion of the yolk supply in those cells which produce the enzyme; on the other hand, the activity of amylase might be controlled by some intracellular feedback mechanism.

The properties of larval and adult amylases are quite similar, and there is little reason to suppose that the veliger contains a different protein from that of the adult. Amylase is a relatively simple protein which has been crystallized from several sources, and it appears feasible to obtain highly purified preparations from both larval and adult oysters. A further characterization of purified enzyme from both sources might reveal subtle differences which were not detected in the present study.

The data indicate that amylase reaches a high level of activity prior to the intake of any food by the larva. Amylase may be one of a number of digestive enzymes which are produced or activated simultaneously just before the feeding stage is reached. Such increases in amylase and other digestive enzymes have also been reported in the amphibian, *Siredon* (*cf.* Løvtrup, 1959). It seems likely that the survival of larvae such as those of the oyster, which have little yolk reserve, will depend to a large extent on the types and specificities of the digestive enzymes which are present at the time that feeding begins. From the point of view of both the developmental physiologist and the ecologist it would be of value to determine what internal and external factors influence the activities of these enzymes during endodermal differentiation and during the early period of feeding.

The authors are indebted to Mr. Robert Mentzer for his technical assistance during this investigation.

Summary

Alpha amylase activity is absent from developmental stages of the oyster up to the straight-hinge veliger. Its development after this stage parallels the development of the intestine. After reaching a maximum at 37 hours, the enzyme drops sharply in activity, presumably because of starvation. The larval enzyme has the same pH optimum and substrate specificity as that of the adult, but the energy of activation is somewhat lower than that of the adult.

LITERATURE CITED

Bernfeld, P., 1955. Amylases, α and β. Methods in Enzymology, I: 149–158. Ed. by S. P. Colowick and N. O. Kaplan. Academic Press, New York.

Brachet, J., 1960. Biochemistry of Development. Pergamon Press, New York.

Løvtrup, S., 1959. Biochemical indices of embryonic differentiation. *In:* Biochemistry of Morphogenesis, pp. 105–125. Proceedings of the Fourth International Congress of Biochemistry. Ed. by W. J. Nickerson. Pergamon Press, New York.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

Moog, F., 1958. Enzymes: formation and growth. *In:* Symposium on Embryonic Nutrition, pp. 87–102. Ed. by D. Rudnick. University of Chicago Press.

Moog, F., 1959. The adaptations of acid and alkaline phosphatases in development. *In:* Cell, Organism and Milieu, pp. 121–155. Ed. by D. Rudnick. Ronald Press, New York.

Prosser, C. L., and F. A. Brown, Jr., 1961. Comparative Animal Physiology, p. 128. W. B. Saunders, Philadelphia.

van Weel, P. B., 1961. The comparative physiology of digestion in molluscs. *Amer. Zool.*, **1**: 245–252.

Yonge, C. M., 1926. Structure and physiology of the organs of feeding and digestion in *Ostrea edulis*. *J. Mar. Biol. Assoc.*, **14**: 295–386.

PHYSIOLOGY OF INSECT DIAPAUSE. XIII. DNA SYNTHESIS DURING THE METAMORPHOSIS OF THE CECROPIA SILKWORM

BLAIR BOWERS[1] AND CARROLL M. WILLIAMS[2]

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Ten micrograms of ecdysone injected into a brainless, diapausing Cecropia pupa results in the termination of diapause within 36 hours (Williams, 1954). The same result is seen when the injection is into an isolated pupal abdomen. Without the injection, neither the brainless pupa nor the isolated abdomen would be able to initiate adult development. The abdomen will not develop because of the absence of prothoracic glands. The brainless pupa will not develop because the prothoracic glands, though present, are unable to secrete ecdysone in the absence of the tropic stimulus of brain hormone. Yet almost overnight, the injection of a minute amount of ecdysone terminates the "permanent" diapause and swiftly mobilizes the dormant cells and tissues in a complex and coordinated developmental response.

Manifestly, this experiment is a caricature of what goes on within diapausing pupae in the spring of the year. The brain hormone is secreted and drives the prothoracic glands; then over a period of a week or two, the pupa gives *itself* the ecdysone injection.

The termination of diapause, whether naturally achieved or experimentally induced, focuses attention on endocrinological events at the cellular level. How does ecdysone, after months of developmental standstill, restore to the diapausing cells their capacity to grow?

The present approach to the problem has been motivated by our previous unpublished cytological studies of diapausing and post-diapausing tissues. In preparation of diapausing tissues one cannot fail to be impressed by the absence of any signs of mitotic activity—a generalization to which the hemocytes and spermatogonia seem to constitute the only exceptions. By contrast, the action of ecdysone and the initiation of adult development are accompanied in the vast majority of tissues by what is little short of a mitotic explosion.

Notwithstanding this fact, there are cogent reasons for disbelieving that ecdysone acts by stimulating cell division. For example, the response of most larval tissues to ecdysone includes growth by a combination of mitosis and cell enlargement, the latter being accompanied by chromosome replication. Indeed, it will be recalled that in the higher Diptera and Hymenoptera, the growth of larval tissues seems to be solely by cell enlargement, including chromosomal replication. So, in cytological terms, it is chromosomal replication rather than cell division which seems to distinguish between growing and diapausing cells.

---

[1] Predoctoral Fellow of the National Cancer Institute of the U. S. Public Health Service during this investigation.

[2] This study was supported, in part, by a grant from the National Institutes of Health.

In the present investigation we have sought to test this hypothesis by means of an autoradiographic study of DNA synthesis in diapausing and non-diapausing Cecropia and Cynthia silkworms.

METHODS

1. *Experimental animals*

The experiments were performed on male larvae and pupae of *Hyalophora cecropia* (L.) and male pupae of *Samia cynthia* (Drury). The Cecropia were reared on wild-cherry trees; the Cynthia were obtained from dealers.

Large injuries were made on diapausing Cynthia pupae as described by Harvey and Williams (1961).

2. *Injections*

Fifth instar larvae were anesthetized with carbon dioxide and a dorsal tubercle of the sixth abdominal segment was excised with scissors. The injection of thymidine was made through the tubercle from a 0.25-ml. hypodermic syringe *via* a 27-gauge needle, and the opening immediately sealed with melted wax. Each larva received 4 or 5 μc. of tritiated thymidine per gram live weight. The caterpillars were kept at room temperature for 6 to 27 hours before fixation.

Pupae were injected, without anesthesia, in the mesothoracic dorsum just lateral to the mid-line, and the puncture wound sealed promptly with melted wax.

TABLE I

Summary of animals studied

Animal # Cecropia	Stage	H^3-thymidine per g. body wt.		Time from injection to fixation (hours)
		μc.	mμmoles	
L1	Late 5th instar (4.1 g.)	5	3	6
L2	Late 5th instar (4.4 g.)	5	3	18
L3	Late 5th instar (4.7 g.)	4	2	27
L4	Spinning outer cocoon	4	2	19
L5	Early prepupa (finished inner cocoon; eye epidermis had retracted)	4	2	19
P1	6–8 days after larval-pupal molt	4	2	19
P2	7 days after larval-pupal molt	6	2	18
P3	14 days after larval-pupal molt	6	2	18
P4	Diapause: 2–3 months after pupation	7	8	19
P5	Diapause: 5 months after pupation (Q_{O_2}:15.5)*	4	2	18
P6	Diapause: 5 months after pupation (Q_{O_2}:9.6)*	4	2	18
P7	Diapause (Q_{O_2}:9.1)* placed at 6° C. for 10 weeks; fixed at 6° C.	6	7	1680
P8	Diapause (Q_{O_2}:10.5)* placed at 6° C. for 10 weeks; fixed at 6° C.	6	4	1680
P9	Chilled 10 weeks at 6° C. Injected after return to 25° C.	6	2	6
P10	Chilled 10 weeks at 6° C. At 25° C. for 7 days (Q_{O_2}:57)*	6	2	27
P11	Chilled 10 weeks at 6° C. At 25° C. for 11 days (Q_{O_2}:55)*	6	2	27
A1	Reached day 0 of adult development at 6° C.; injected when removed to 25° C.	4	2	19
Cynthia				
P12	Diapause: 2–3 months after pupation	10	11	27
P13	Diapause: 6–7 months after pupation	10	11	2880
A2	Day 2 of adult development	4	4	18
A3	Day 2 or 3 of adult development	6	7	27
A4	Day 3 of adult development	10	11	18
I1	Three days after injury	4	1	18
I2	Six days after injury	4	1	18
I3	Six days after injury	4	1	18
I4	Nine days after injury	4	1	18

* Oxygen consumption measured in Warburg manometers, usually for a five-hour period; expressed as μl. oxygen consumed per gram live weight per hour.

The volume of each injection was 0.01 to 0.02 ml., and each pupa received 4 to 10 μc. of the thymidine. The pupae were stored at 25° C. or at 6° C. until fixed. Table I summarizes the status and treatment of the 26 animals that were studied.

3. Thymidine

The tritium-labeled thymidine was from three batches: New England Nuclear Corp. (Boston, Mass.), with a specific activity of 890 millicuries per millimole; Schwarz Laboratories, Inc. (Mt. Vernon, N. Y.), with a specific activity of 1600 mc./m*M*; and Schwarz, with a specific activity of 3000 mc./m*M*.

4. Fixation and embedding

In the early experiments, the wings and testes of the injected individuals were dissected from unanesthetized animals and fixed in one of several standard fixatives: Bouin, Helly, Carnoy (6:3:1), Flemming (0.4 gram osmic acid, 0.72 gram chromic acid in 100 ml. water), or 95% ethanol-glacial acetic acid (3:1). One of the paired structures was fixed in each of two solutions.

In later experiments, entire animals were fixed in 10% aqueous acrolein at room temperature for two hours, the cuticle being sliced to facilitate entrance of the fixative. As the tissue hardened, the initial incisions were extended to transect the animals. They were post-fixed in Methyl Formcel (Celanese Corp., Framingham, Mass.) for 12 to 24 hours and dehydrated in methanol. Two animals (P2, P3) were fixed in 10% acrolein at 0° C. and dehydrated in ethylene glycol monomethyl ether and methanol, as described by Feder (1960).

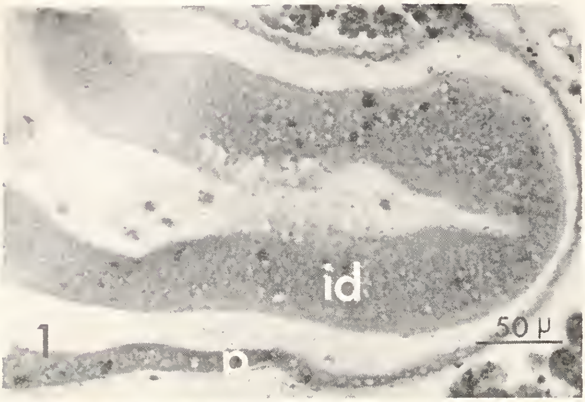
The fixed material was embedded in paraffin wax (Fisher Tissuemat, M.P. 58–60° C.) or in polyester wax (Sidman *et al.*, 1961). The sections were routinely cut at six micra. Since about 90% of the tritium radiation is absorbed in 1.2 μ of tissue (Fitzgerald *et al.*, 1951), these sections were "infinitely thick" with respect to tritium disintegrations.

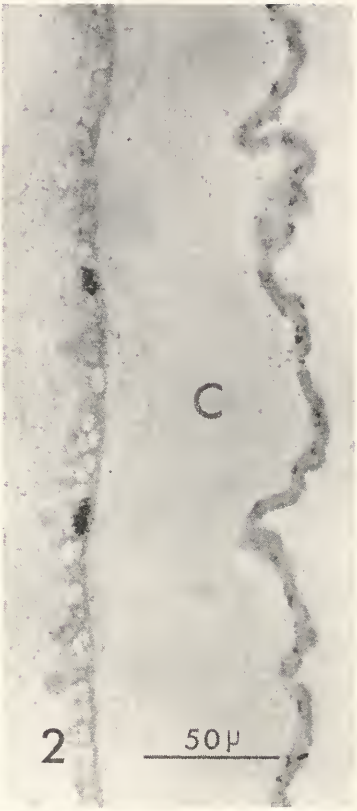
5. Preparation of radioautograms

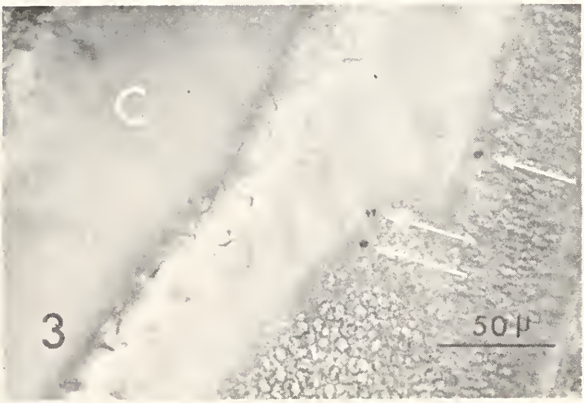
Radioautograms were prepared as described by Messier and Leblond (1957). The slides were coated with Kodak Nuclear Emulsion, Type NTB 3; the latter had a thickness of approximately five micra.

After exposure times of 1 to 6 weeks at −20° C., the radioautograms were developed by $1\frac{1}{2}$ minutes' immersion in Kodak developer D-72, followed by 10 minutes in Eastman formula F-5 acid fixing bath. The slides were rinsed at least 45 minutes in 4 or 5 changes of tap water. They were then taken directly to the staining solution. The stain routinely employed in this study was 1% aqueous toluidine blue, but some slides were stained by the Feulgen procedure (Stowell, 1945) before coating. The sections were finally dehydrated, cleared, and mounted in damar.

The dose of isotope was always high enough to give unequivocal radioautograms (4 to 40 grains per nucleus) after seven days' exposure. In each case some sections were exposed for 14 and 28 days, to check for very low levels of labeling. The cells were scored either as "labeled" or "unlabeled."







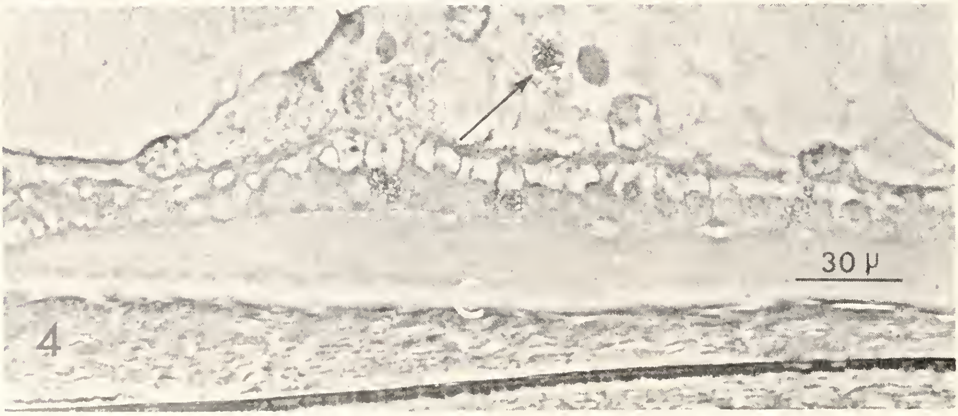


FIGURE 1. Radioautogram of a section of wing disc from a late fifth instar Cecropia. Nuclei are unstained. id, imaginal disc; p, peripodal membrane.

FIGURE 2. Radioautogram of a section of wing of a Cecropia pupa one week after pupal ecdysis. Presence of radioactive nuclei indicates DNA synthesis by epidermal cells at this time. Note the accumulations of silver grains in the cuticle (C), probably due to reducing compounds such as polyphenols.

6. *Specificity of thymidine as a DNA precursor*

In sections of tissues fixed in Bouin's or Helly's fluids, the extraction of RNA and unincorporated precursors of the nucleic acids was accomplished by hydrolysis with 10% perchloric acid at 20° C. for two hours (Woods and Taylor, 1959; Atkinson, 1952).

DNA was selectively extracted from sections fixed in 95% ethanol:glycol acetic acid (3:1) by means of crystalline deoxyribonuclease (Nutritional Biochemical Corp., Cleveland, Ohio). The enzyme was prepared in Gomori's Tris-maleic acid buffer (Gomori, 1952), adjusted to 0.02 *M* with respect to $MgSO_4$ and to pH 5.95 with 1 N NaOH (McDonald, 1955). The concentration of the solution was 5 mg. of enzyme in 100 ml. of buffer. Slides were incubated for 18 hours at 37° C. (Amano *et al.*, 1959). Control slides were incubated with buffer in the absence of enzyme. After incubation, both the DNase-digested and the control slides were rinsed in distilled water, hydrolyzed 8 minutes in 1 N HCl at 60° C., and stained with Schiff reagent. The slides were coated with emulsion by the procedure described above.

Hydrolysis in 10% perchloric acid did not remove the radioactivity localized over the nuclei; this indicates that the label was in polymerized DNA. Slides incubated in DNase showed no Feulgen staining and gave no radioautogram. The control slides, consisting of sections adjacent to those digested, retained their nuclear-localized radioactivity and the capacity to stain with Schiff reagent.

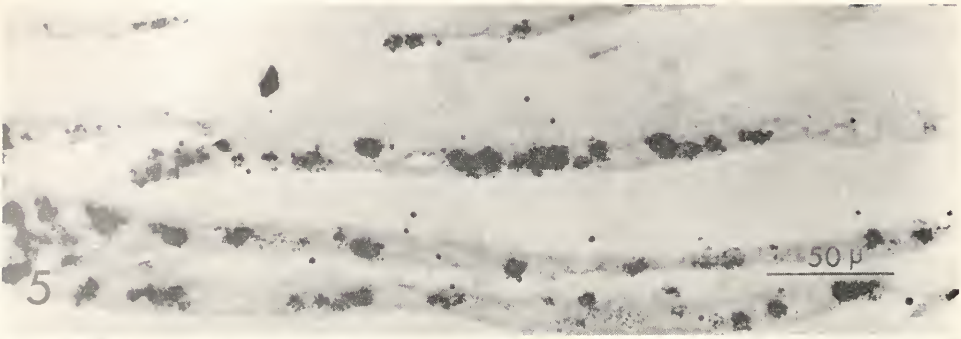
Experimental Results

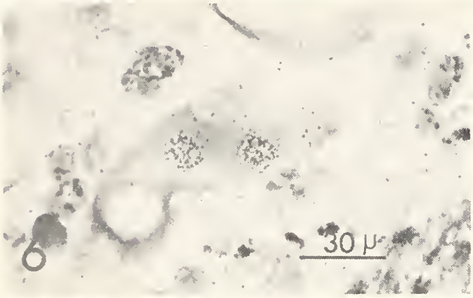
1. *The wing anlagen*

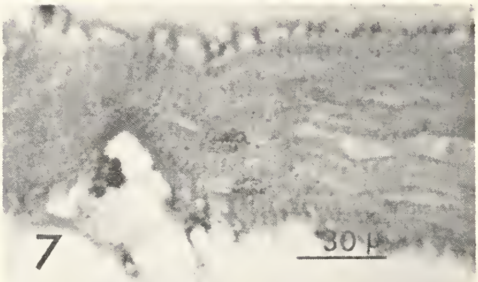
In larval Lepidoptera the anlagen of the future wings consist of two pairs of imaginal discs situated on each side of the meso- and metathorax just internal to the epidermis. Each disc takes the form of a flattened pouch of pseudostratified epithelium, which is invaginated into and surrounded by a thinner envelope of epithelium, the latter forming the so-called peripodal sac. During successive stages of larval life the wing anlagen undergo a steady increase in size; their dry mass, in fact, shows the same relative growth rate as does the larva itself (Williams, unpublished measurements). At the onset of the prepupal period—a stage recognizable by the retraction of the pigment granules of the ocelli (Kühn and Piepho, 1936)—the wing discs undergo a sudden spurt in growth and are drawn to the outside of the body to lie beneath the loose cuticle of the final larval instar. The shedding of this cuticle at the time of the pupal ecdysis reveals the prominent wing-pads of the pupa itself. Each consists of a flattened sac of epithelium. Unlike the imaginal discs, the surfaces of pupal wings are cuticularized; this is particularly true of the outer exposed surface of the forewings.

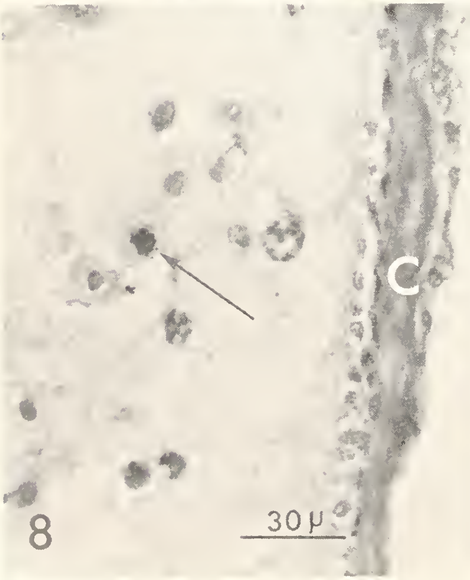
Figure 3. Epithelium in the head region of a Cecropia pupa one week after the pupal ecdysis. Arrows indicate dividing epithelial cells. This is a stained section and not a radioautogram. C, cuticle.

Figure 4. Radioautogram of a section of a wing of a Cecropia pupa shortly before the visible initiation of development. In this phase contrast image, some of the silver grains appear as bright spots. The epidermis still adheres tightly to the cuticle (C). Arrow indicates blood cell which shows incorporation.









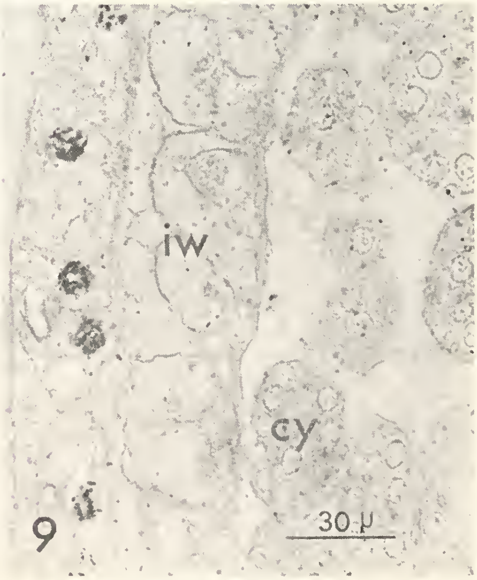


FIGURE 5. Radioautogram of a section of wing of a Cecropia pupa at "day 1" of adult development. Nearly half of the epidermal cells have incorporated tritiated thymidine. The slide was exposed for four weeks; hence the very heavy deposits of silver.

FIGURE 6. Radioautogram of a section of a Cecropia pupa before the visible initiation of adult development. The nuclei of two tracheoblasts have incorporated tritiated thymidine.

After the sclerotization of their cuticle, the pupal wings undergo no obvious change during the true pupal period. In non-diapausing insects this stage lasts only a few days before adult development begins. However, in diapausing pupae, such as that of the Cecropia silkworm, the pupal stage persists for about 8 months. Then, with the initiation of adult development, the epidermis of the wings retracts from the overlying pupal cuticle and enters at once into a swift and complicated program of differential cell divisions which has been described in such impressive detail by Henke and his students (for summary, see Kühn, 1955).

In the present study, the incorporation of tritiated thymidine into the DNA of the nuclei of the cells of the wing anlagen was studied in a series of 17 Cecropia ranging from the fifth larval instar to an early stage in adult development.

In line with the rapid growth of the wing during the larval and prepupal periods, incorporation of label (Fig. 1), as well as mitotic figures, was detected in all of a series of five fifth-instar larvae described in Table I. This was true of the disc itself as well as the surrounding peripodal sac. In all cases the labeled cells and the mitotic divisions appeared to be in localized clusters scattered through the wing tissues. Mitotic activity was highest in the discs of a mature larva which was in process of spinning its cocoon. Incorporation of label was maximal in the early prepupal stage where about 17% of cells showed DNA synthesis within a period of 19 hours.

A surprising finding was a continuation of DNA synthesis in the wings of pupae seven days after the pupal ecdysis (see Fig. 2). Synthesis continued at this stage despite the apparent absence of mitotic divisions. Two weeks after the pupal ecdysis, all incorporation of label ceased. Four diapausing Cecropia pupae (P3, P4, P5, and P6) showed no trace of DNA synthesis after storage at 25° C. for as long as five months.

Exposure to low temperatures is known to potentiate the initiation of adult development by restoring the brain's endocrine activity. Thus, after ten weeks at 6° C., Cecropia pupae initiate development within several weeks after their return to 25° C. (Williams, 1956). DNA synthesis at the low temperature was studied in two pupae (P7, P8) which were injected with tritiated thymidine and immediately placed at 6° C. Ten weeks later, the animals were sacrificed without rewarming them. The wing epithelia showed no incorporation of label during this long exposure. In a third pupa (P9) the injection of label was postponed until the end of the ten weeks of chilling. After six hours at 25° C., the wings of the pupa likewise showed no DNA synthesis.

Of particular interest are two further pupae of this same series. These were subjected to ten weeks of chilling but the injection of label was further postponed

FIGURE 7. Radioautogram of a section of a Cecropia pupa before the visible initiation of adult development. Incorporation of triated thymidine is evident in the nuclei of an intersegmental muscle of the abdomen.

FIGURE 8. Radioautogram of a section of a wing of a Cynthia pupa injected with tritiated thymidine six days after an integumental injury. The epidermal cells show no incorporation of label; one blood cell (arrow) in this field shows label.

FIGURE 9. Radioautogram of a section of a testis of a Cynthia pupa at "day 2–3" of adult development. Five nuclei of the outer wall of the testis are radioactive. None of the spermatocytes are labeled. iw, inner wall of testis; cy, spermatocyte cyst.

after their return to 25° C. P10 was stored at 25° C. for seven days, at which time its Q_{O_2} had increased to 57 μl. per gram live weight per hour. P11 was stored at 25° C. for eleven days and showed a Q_{O_2} of 55. Both animals were then exposed to tritiated thymidine for 27 hours at 25° C. Despite the fact that neither pupa showed any obvious trace of the initiation of adult development, approximately 14% of the cells of the wings were labeled (Fig. 4).

After very prolonged storage at 6° C., Cecropia pupae show a retraction of facial and wing epidermis and other signs indicating the initiation of adult development at the low temperature (Williams, 1956). One pupa of this type was studied (A1). This animal was injected with isotope and placed at 25° C. for 19 hours. The wings showed numerous mitotic divisions and a high percentage of labeled cells (up to 47% in certain sections). A radioautographic section of a wing of this animal is shown in Figure 5.

The above-mentioned results were confirmed in a less extensive study of the Cynthia silkworm. Here, no incorporation of label was found in the wing epithelium of two diapausing pupae stored at 25° C. (P12, P13). By contrast, three animals (A2, A3, A4), ranging from "day 0" to "day 3" of adult development at the time of injection, showed frequent mitotic figures and substantial incorporation of label.

2. *The testes*

The male gonads are paired, kidney-shaped organs lying dorsally on each side of the heart in the fifth and sixth abdominal segments. Each testis consists of four compartments surrounded and bound into one complex by a testicular capsule. The gross morphology of the testis, unlike that of the ovary, does not change during metamorphosis.

The capsule of the testis consists of two distinct layers (Fig. 9), the inner of which is continuous with the three septa which subdivide the testis into four compartments. The cells of the inner layer seem to function as a storage tissue during the pupal period. The entire capsule is heavily laced with trachea and tracheoles which supply the cells therein and pass into the lumen to supply the germinal cells.

Primordial germ cells appear in the cavities of the testis at an early embryonic stage. They become localized at the expanded blind end of each of the four compartments and, by repeated mitotic divisions, give rise to spermatogonia throughout larval life. The spermatogonia become isolated in spherical clusters of 16 to 24 cells and enveloped by follicle cells (Cook, 1910). The latter are mesothelial cells which envelop the spermatogonia as a simple squamous sheath. The whole complex forms the so-called "germinal cyst." By the late fifth instar, the four chambers of each testis are packed with these cysts. Approximately one-third contain spermatogonia, one-third contain spermatogonia in synapsis, and one-third contain primary spermatocytes. All types of pre-meiotic cysts persist in the testes in varying proportions until the pupa terminates diapause and initiates adult development. At this time the primary spermatocytes undergo meiosis and proceed with spermiogenesis. During the 21-day period of adult development all of the germinal cysts mature into spermatozoa.

During larval life the testes grow at the same relative rate as the larva itself. But, unlike the wing anlagen, which undergo a spurt of growth during the prepupal period, the testes respond to pupation as if it were just another larval molt (Williams, unpublished measurements).

In the present study we found that tritiated thymidine was incorporated by the spermatogonia, but not by primary spermatocytes or any subsequent stage. The spermatogonia incorporated the label at each period in the life-cycle—even during pupal diapause. In like manner, mitotic figures were evident in the spermatogonia in each animal which was examined, including the diapausing pupa. No incorporation of label was observed in the follicle cells at any stage from mature larva to early adult development.

If attention is directed to the cells which comprise the capsule and walls of the testis, here, again, at least an occasional cell was found to incorporate label at all stages including the diapausing pupa. The synthesis of DNA was particularly prominent in the outer layer of the capsule in the early prepupa (L5) and at the outset of adult development (A1, A3) (Fig. 9).

In summary, we find that the spermatogonia show a contrasting behavior to that of the wing epithelium, in that the synthesis of DNA is not completely shut off during the pupal diapause. The cells of the testicular wall show a maximum of DNA synthesis at the same periods in which the wing epithelium synthesizes DNA; in addition, a few cells incorporate label during diapause.

3. *Blood cells*

These cells incorporated thymidine at every stage surveyed. Labeled blood cells were observed in the lumen of the wings and legs, in the head, in the abdomen—in short, throughout the entire animal. The percentage of labeled cells varied from 1% to 10% without any discernible relation to developmental stage. Mitotic divisions were seen in a prepupa, a diapausing pupa, and in a pupa just prior to initiation of adult development.

4. *Midgut*

The midgut of the pupa consists of a columnar epithelium with a brush border, subtended by layers of circular and longitudinal muscles. The columnar cells are derived from crypts of regenerative cells at the base of the epithelium (Wigglesworth, 1950). As might be expected, among the epithelial cells only the regenerative ("crypt") cells showed any incorporation of isotope. Labeling of cells was not observed in an animal one week after the pupal molt; but two weeks after pupation, and in diapausing animals stored at 25° C. or at 6° C., a few of these cells were labeled. The largest number of radioactive regenerative cells was found in an animal chilled for ten weeks and returned to room temperature for six hours (P9). During the early stages of adult development (P10, P11), the crypt cells had increased in number over the diapause condition, but only rarely showed any evidence of DNA synthesis.

The muscular layer of the midgut is interlaced with trachea and highly infiltrated by blood cells. This region showed a few cells incorporating at each stage, but the radioactivity observed here can most probably be attributed to blood cells.

5. *Other tissues*

The epidermal cells of the pupal head and antennae showed mitotic divisions and incorporation of label one week after the pupal ecdysis (Fig. 3). However, within the following week these activities disappeared (P3) and remained absent throughout the pupal diapause (P4, P5, P6, P7, P8, P12, P13).

Synthesis of DNA was also detected in the thoracic myoblasts and cells of the central nervous system one week after the pupal ecdysis (P2). Within the following week the myoblasts showed no further synthesis, whereas an occasional cell of the central nervous system continued to incorporate label. All these activities came to a halt after the onset of pupal diapause, *i.e.*, approximately two weeks after the pupal molt.

Prior to the retraction of the pupal wing epidermis, signaling what has been called the "zero day of adult development," the resumption of DNA synthesis was already evident (P10, P11) in the cells of the central nervous system, the tracheoblasts (Fig. 6), the myoblasts of the abdominal segments, and the nuclei of the intersegmental muscles of the pupal abdomen (Fig. 7). The cells of the fat body did not resume DNA synthesis until after the zero day of adult development (A1).

The cells of the Malpighian tubules showed no incorporation of label at any stage that was examined.

6. *Effects of integumentary injury to diapausing pupae*

The effects of large integumentary injuries were studied on four diapausing Cynthia pupae at 25° C. The animals were injured and tritiated thymidine was injected 3, 6, and 9 days later; after 18 hours, the pupae were sacrificed and examined.

No resumption of DNA synthesis was detectable in the wing epidermis (Fig. 8), the abdominal epidermis, or the intersegmental muscles of the abdomen. However, radioactive nuclei were encountered in the thoracic myoblasts, in an occasional regenerative cell of the midgut and in a few epidermal cells in the immediate vicinity of the wound.

The most spectacular effect of injury was on the blood cells. After 18 hours of exposure to label, about 30% showed DNA synthesis. The labeled cells accumulated in largest numbers at or near the wound, but were also found throughout the animal as a whole. Mitotic figures were seen in all the injured pupae, although they were more numerous in the animal nine days after injury.

DISCUSSION

1. *The chronology of DNA synthesis*

a. *The pre-diapause pupa*

During the first two weeks after the pupal ecdysis the rate of oxygen consumption declines to a low level which then persists during the months of pupal diapause (Schneiderman and Williams, 1953). The entire endocuticle, making up about 80% of the mature pupal cuticle (Passonneau and Williams, 1953), is secreted during this two-week period (Harvey and Williams, unpublished observations)—a finding which documents a continuation of synthetic operations within the freshly pupated insect.

In the present study this conclusion was further reinforced by the finding that DNA synthesis and mitotic divisions continue in many tissues immediately after the pupal molt. This suggests the possibility that, in Cecropia at least, a limited progress toward differentiation of the adult may occur at this time.

b. The diapausing pupa

The onset of diapause may be dated from approximately 10 to 14 days after pupal ecdysis at 25° C. By the fourteenth day, DNA synthesis could no longer be detected in the epithelial cells of the hypodermis. This condition of arrested synthesis then persists during the months of pupal diapause, irrespective of whether the pupae are stored at 25° C. or 6° C.

The present investigation has identified two types of cells which are exceptional in that both DNA synthesis and mitosis apparently continue after the pupa is firmly established in diapause. These cells include the hemocytes and the spermatogonia. Labeled nuclei were occasionally observed among the cells of the testicular walls and the regenerative cells of the midgut epithelium, but no mitotic divisions were seen in these tissues.

c. Injured diapausing pupae

The full significance of the just-mentioned observations is not wholly clear, for the reason that the injection of tritiated thymidine constitutes an injury which in itself swiftly mobilizes many of the metabolic and synthetic activities of diapausing pupae (Shappirio, 1958, 1960; Telfer and Williams, 1960; Skinner, 1960, 1963; Laufer, 1960; Stevenson and Wyatt, 1962). Therefore, the experiments performed on diapausing Cynthia pupae subjected to large integumentary injuries are of particular interest and importance.

With few exceptions those types of cells which show no DNA synthesis in injected but otherwise uninjured pupae continued to show no synthesis when the injection was preceded by a large injury. A few thoracic myoblasts and an occasional epidermal cell in the immediate vicinity of the wound constitute the exceptions to this generalization.

Attention is now directed to those types of cells which show a low level of incorporation of injected thymidine, *e.g.*, cells of the testicular wall. Only in the case of the hemocytes did large integumentary injuries provoke any noteworthy increase in the number of cells showing DNA synthesis.

Thus, we find that injury to a diapausing pupa is a potent stimulus for DNA synthesis in the hemocytes. For all other tissues it seems, at most, to be an extremely feeble stimulus.

d. The post-diapause pupa

After ten or more weeks at 6° C., the pupal brain regains its ability to secrete the brain hormone which promotes the initiation of adult development by "turning on" the prothoracic glands. At 6° C. the secretion and translocation of brain hormone occur slowly and are complete after approximately ten months (Williams, 1956). Then, despite the low temperature, adult development progresses as far as retraction of the wing epidermis from the overlying pupal cuticle.

If the period of chilling is less prolonged, adult development begins only after the pupa has been placed at higher temperatures for one to two weeks. Approximately one week prior to the retraction of wing epidermis, the rate of oxygen consumption begins to increase.

As pointed out by Schneiderman and Williams (1953), this gradual increase in respiration indicates that the biochemical events associated with the termination of diapause are initiated at least one week prior to any morphological sign of development. The results of the present study are in full accord with this interpretation. During the week prior to the visible initiation of adult development, DNA synthesis is resumed or accelerated in the vast majority of tissues. The earliest response is seen in the regenerative cells of the midgut, followed shortly thereafter by the epidermal cells of the wings, the intersegmental muscles of the abdomen, tracheoblasts, myoblasts, and the cells of the central nervous system.

c. The developing adult

The detachment and retraction of the epidermis occur first in the region of the eye lobes and genitalia and then swiftly spread to the wings. It is clearly evident in the legs by the second day of adult development at 25° C.

Though the majority of tissues show a resumption or acceleration of DNA synthesis before the retraction of the epidermis, the fat body is exceptional in that its response is delayed until the first few days of adult development.

2. Hormones and DNA synthesis

a. Biochemical changes

DNA replication may be included in a long list of synthetic activities which subside immediately prior to the onset of pupal diapause, remain at a low level for months thereafter, and accelerate during the termination of diapause and initiation of adult development (Gilbert and Schneiderman, 1961; Harvey, 1962; Wyatt, 1959, 1962, 1963). As mentioned earlier, this same ebb and flow is seen in the overall metabolism in terms of the rate of oxygen consumption of the animal as a whole.

All these systematic alterations are of special interest because they proceed in parallel with the changing concentration of prothoracic gland hormone (ecdysone). By contrast, they show no clear correlation with juvenile hormone, for the latter declines prior to pupation and is virtually absent during the early phases of adult development (Williams, 1961).

b. Ecdysone vs. the "injury factor"

There seems little reason to doubt that, in one way or another, previously described biochemical and metabolic changes associated with the termination of diapause are directly or indirectly promoted by ecdysone. Yet, strange to say, all of these changes accompanying the action of ecdysone and the initiation of adult development can take place in the absence of ecdysone and without any trace of adult development. It is only necessary to make an injury in the pupal integument.

It is not our present purpose to enter into a detailed discussion of the injury response or the "injury factor" which is apparently released from the wound. These matters have already been subjected to detailed study and review (Shappirio, 1960; Harvey and Williams, 1961; Wyatt, 1963). Suffice it to say that no previously described chemical or metabolic criterion has permitted one to distinguish between a wounded diapausing pupa and a developing moth. Evidently, the biochemical response to ecdysone includes one or more effects which are necessary for development but which cannot be duplicated in response to the injury factor. Therefore, the biochemical difference between injured diapausing pupae and developing adults is a matter of central significance to any comprehensive theory of the mode of action of ecdysone.

The present study has directed attention to what appears to be the first such difference that has been recognized. For most cell types, the prothoracic gland hormone constitutes a potent stimulus for DNA synthesis, whereas the injury factor does not.

c. *Ecdysone and DNA synthesis*

Among the various tissues here examined, there appear to be at least three categories of response, in terms of DNA synthesis, to development and to injury:

Pupal hemocytes show a specific response to large injuries by enhanced DNA synthesis and by accumulation at the site of injury. This finding is reasonable in view of the active role which hemocytes play in wound healing (Wigglesworth, 1937; Smith and Schneiderman, 1954; Lea and Gilbert, 1961). An increase in circulating blood cells after injury has been previously noted by several investigators (Harvey and Williams, 1961; Lea and Gilbert, 1961), and the results here indicate that this is a true multiplication and not simply a mobilization of sedentary cells. Pupal hypodermal cells in the immediate vicinity of the wound also show a specific response to injury by DNA synthesis in a limited number of cells, as previously observed by Davis and Schneiderman (1960).

Certain tissues, such as thoracic myoblasts, the regenerative cells of the midgut, and the tracheoblasts, exhibit a "metastable" response. They respond most markedly at times of high ecdysone titer, *i.e.*, at the pupal molt and at the beginning of adult development. But they also apparently respond to states of heightened metabolism (*e.g.*, injury) by sporadic synthesis of DNA. Although spermatogonia appear to continue a slow rate of DNA synthesis and cell division throughout diapause, *meiotic* divisions are rarely seen in an uninjured diapausing pupa. Such divisions are frequently seen in sections of testes taken from animals during the pupal molt, at the beginning of adult development, and after large injuries—that is, at times of heightened metabolism. The cysts which show meiosis at pupation and after injury degenerate without forming spermatozoa (Bowers, unpublished observations).

Finally we may note a third category of tissues—the hypodermis (with the exception of the margins of a wound) and the abdominal intersegmental muscles—which seem to synthesize DNA only when ecdysone is being secreted. Thus these tissues appear to respond specifically to the presence of ecdysone. Similar findings have been reported for *Rhodnius* (Wigglesworth, 1963) where ecdysone is necessary for the "activation" of epidermal cells and intersegmental muscles, but is not necessary for the activation of fat body or hemocytes. In the case of Cecropia, however, the analogy does not extend to fat body, since this tissue was not observed to synthesize DNA until after visible initiation of development.

It seems not unlikely that detailed and quantitative studies of DNA synthesis in injured pupae and in developing adults may lead to a more intimate understanding of the mode of action of ecdysone.

SUMMARY

1. DNA synthesis at stages in the life-cycle of the Cecropia and Cynthia silkworms was examined by radioautographic survey of the incorporation of tritiated thymidine.

2. In sections of pupae previously injected with tritiated thymidine, incorporation of the nucleotide into DNA, as well as cell division, was observed in several tissues as late as one week after pupation.

3. In diapausing pupae, incorporation of thymidine occurred in spermatogonia, hemocytes, a few midgut regenerative cells, and a few cells of the testicular walls. Neither incorporation nor mitotic figures were observed in diapausing epidermal tissues.

4. Large epidermal injury increased DNA synthesis in the blood cells of diapausing pupae and induced incorporation in a few epidermal cells in the immediate vicinity of the wound. Epidermal cells outside the wound periphery did not incorporate thymidine after injury.

5. A generalized incorporation of thymidine was observed in synchrony with the termination of pupal diapause. The first cells to show increased incorporation were the regenerative cells of the midgut, followed by cells of the epidermis, muscles, nerves, tracheoblasts, and, ultimately, the fat body. The incorporation in the epidermis precedes and then accompanies the extensive cell division associated with adult differentiation.

6. Lack of DNA synthesis in the epidermal tissues of injured diapausing pupae appears to be the first biochemical or metabolic criterion so far recognized that permits one to distinguish between an injured pupa and a developing adult.

LITERATURE CITED

AMANO, M., B. MESSIER AND C. P. LEBLOND, 1959. Specificity of labeled thymidine as a DNA precursor in radioautography. *J. Histochem. Cytochem.*, **7**: 153–155.

ATKINSON, W. B., 1952. Differentiation of nucleic acids and acid mucopolysaccharides in histologic sections by selective extraction with acids. *Science*, **116**: 303–305.

COOK, MARGARET H., 1910. Spermatogenesis in lepidoptera. *Proc. Acad. Nat. Sci. Philadelphia*, **62**: 294–327.

DAVIS, R. P., AND H. A. SCHNEIDERMAN, 1960. An autoradiographic study of wound healing in diapausing silkworm pupae. *Anat. Rec.*, **137**: 348.

FEDER, NED, 1960. Some modifications in conventional techniques of tissue preparation. *J. Histochem. Cytochem.*, **8**: 309–310.

FITZGERALD, P. J., M. L. EIDINOFF AND E. B. SIMMEL, 1951. Tritium in radioautography. *Science*, **114**: 494–498.

GILBERT, L. I., AND H. A. SCHNEIDERMAN, 1961. Some biochemical aspects of insect metamorphosis. *Amer. Zool.*, **1**: 11–51.

GOMORI, G., 1952. Microscopic Histochemistry, Principles and Practice. Univ. of Chicago Press.

Harvey, W. R., 1962. Metabolic aspects of insect diapause. *Ann. Rev. Ent.*, **7**: 57–80.

Harvey, W. R., and C. M. Williams, 1961. The injury metabolism of the Cecropia silkworm. I. Biological amplification of the effects of localized injury. *J. Ins. Physiol.*, **7**: 81–99.

Kühn, Alfred, 1955. Vorlesungen über Entwicklungsphysiologie. Springer-Verlag.

Kühn, A., and H. Piepho, 1936. Über hormonale Wirkungen bei der Verpuppung der Schmetterlinge. *Ges. Wiss. Göttingen, Nachr. Biol.*, **2**: 141–154.

Laufer, H., 1960. Blood proteins in insect development. *Ann. N. Y. Acad. Sci.*, **89**: 490–515.

Lea, M. S., and L. I. Gilbert, 1961. Cell division in diapausing silkworm pupae. *Amer. Zool.*, **1**: 368–369.

Messier, B., and C. P. Leblond, 1957. Preparation of coated radioautographs by dipping sections in fluid emulsion. *Proc. Soc. Exp. Biol. Med.*, **96**: 7–10.

McDonald, Margaret R., 1955. Deoxyribonucleases. *In:* Methods in Enzymology, vol. II (S. P. Colowick and N. O. Kaplan, eds.), Academic Press, New York: pp. 437–477.

Passoneau, J. V., and C. M. Williams, 1953. The moulting fluid of the Cecropia silkworm. *J. Exp. Biol.*, **30**: 545–560.

Schneiderman, H. A., and C. M. Williams, 1953. The physiology of insect diapause. VII. The respiratory metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.*, **105**: 320–334.

Shappirio, D. G., 1958. Injury-induced cytochrome synthesis in diapausing Cecropia silkworms. *Anat. Rec.*, **132**: 506.

Shappirio, D. G., 1960. Oxidative enzymes and the injury metabolism of diapausing Cecropia silkworms. *Annals N. Y. Acad. Sci.*, **89**: 537–548.

Sidman, R. L., P. A. Mottla and N. Feder, 1961. Improved polyester wax embedding for histology. *Stain Tech.*, **36**: 279–284.

Skinner, D. M., 1960. Protein synthesis in the Cecropia silkmoth. *Anat. Rec.*, **138**: 383.

Skinner, D. M., 1963. Incorporation of labeled valine into proteins of the Cecropia silkworm. *Biol. Bull.*, **125**: 165–176.

Smith, R. D., and H. A. Schneiderman, 1954. Healing of epidermal wounds in diapausing pupae of the silkworm. *Anat. Rec.*, **120**: 724–725.

Stevenson, E., and G. R. Wyatt, 1962. The metabolism of silkmoth tissues. I. Incorporation of leucine into protein. *Arch. Biochem. Biophys.*, **99**: 65–71.

Stowell, R. E., 1945. Feulgen reaction for thymonucleic acid. *Stain Tech.*, **20**: 45–58.

Telfer, W. H., and C. M. Williams, 1960. The effects of diapause, development and injury on the incorporation of radioactive glycine into the blood proteins of the Cecropia silkworm. *J. Ins. Physiol.*, **5**: 61–72.

Wigglesworth, V. B., 1937. Wound healing in an insect (*Rhodnius prolixus* Hemiptera). *J. Exp. Biol.*, **14**: 364–381.

Wigglesworth, V. B., 1950. Principles of Insect Physiology. Methuen and Co., London.

Wigglesworth, V. B., 1963. The action of moulting hormone and juvenile hormone at the cellular level in *Rhodnius prolixus*. *J. Exp. Biol.*, **40**: 231–245.

Williams, C. M., 1954. Isolation and identification of the prothoracic gland hormone of insects. *Anat. Rec.*, **120**: 743.

Williams, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull.*, **110**: 201–218.

Williams, C. M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. *Biol. Bull.*, **121**: 572–585.

Woods, P. S., and J. H. Taylor, 1959. Studies of ribonucleic acid metabolism with tritium-labeled cytidine. *Lab. Invest.*, **8**: 309–318.

Wyatt, G. R., 1959. Phosphorus compounds in insect development. *In:* Biochemistry of Insects (L. Levenbook, ed.), Pergamon Press, New York: pp. 161–184.

Wyatt, G. R., 1962. Metabolic regulation in the development of insects. *In:* Control Mechanisms in Respiration and Fermentation (B. Wright, ed.), The Ronald Press, New York: pp. 179–188.

Wyatt, G. R., 1963. Biochemistry of diapause, development and injury in silkmoth pupae. *In:* Insect Physiology (V. J. Brookes, ed.), Biology Colloquium, Oregon State University, Corvallis.

THE PROPERTIES AND SPECIFICITY OF A β-GLUCOSIDASE FROM BLABERUS CRANIIFER[1]

FRANK M. FISHER, JR.

Department of Biology, Rice University, Houston, Texas

The presence of β-glucosidase in insects was first suggested from nutritional experiments (see Lipke and Fraenkel, 1956). Since that time, the enzyme has been demonstrated in a rather large number of insect species. Such enzymatic activity has been demonstrated in crude preparations from *Porcellio* and *Armadillidium*, the pill bugs, and *Periplaneta*, the American cockroach (Newcomer, 1952, 1954, 1956). Koike (1954) investigated the β-glucosidase from *Tenebrio, Bombyx, Dictyoplaca* and *Epicauta;* and by the use of fluorometric methods, Robinson (1956) re-examined the enzyme from *Periplaneta* and *Tenebrio* and compared it with that found in *Notonecta, Locusta* and *Aphis*. Evans (1956) demonstrated the enzyme in preparations from *Calliphora*, using β-phenyl glucoside as a substrate, and confirmed the report by Fraenkel (1940) that the same species could not hydrolyze cellobiose. Fraenkel (*loc. cit.*) also found that adult fly preparations could not hydrolyze α-methyl glucoside, nor were cellobiose or β-methyl glucoside utilized as food substances *in vivo*. The silverfish, *Ctenolepisma*, was shown to possess a β-glucosidase capable of hydrolyzing cellobiose (Lasker and Giese, 1956). The enzyme was also shown to be present in bacteria-free silverfish. Ito and Tanaka (1959) partially purified and characterized the β-glucosidase from *Bombyx* mid gut. Evans and Payne (1960) reported that *Schistocerca* tissue possessed the ability to hydrolyze β-linked carbohydrates. In a survey of the carbohydrases of *Blaberus discoidalis* and *Leucophaea madeirae* (*sic*), Ehrhardt and Voss (1962) described the presence of a β-glucosidase in both roach species, but no characterization was made. Recently, Powning and Irzykiewicz (1962) further investigated the β-glucosidase activity of *Periplaneta* in a comparative study in which they also included *Lycoperdon*, a puffball. The present investigation deals with the partial purification and characterization of a β-glucosidase from *Blaberus craniifer*.

MATERIALS AND METHODS

Experimental material

Cockroaches were maintained in 50-pound lard tins at 24–28° C. on a diet of dog biscuits and ground Purina Lab Chow. Cedar shavings and/or crushed corn cobs were provided as litter, and fresh apples were added periodically.

---

[1] This work was supported in part by a National Institutes of Health, U. S. Public Health Service, Post Doctoral Fellowship and Training Grant No. 2E106.

Reagents

The buffers used in the assay procedures were made with reagent grade chemicals and glass-distilled water. The following buffers were used: (1) phthalate: NaOH, (2) citrate, (3) citrate: phosphate, (4) phosphate, (5) Tris (hydroxymethyl) aminomethane (hereinafter referred to as Tris): HCl, (6) Tris: maleate, and (7) glycine: NaOH. Substrates were also dissolved in glass-distilled water. Alpha-alpha-trehalose, β-methyl-D-glucoside, α-methyl-D-glucoside, α- and β-melibiose, p-nitrophenyl-β-D-glucoside (PNPG), o-nitrophenyl-β-D-galactopyranoside (ONPGal), and gentiobiose were obtained from California Corporation for Biochemical Research. Arbutin and amygdalin were purchased from K and K Laboratories. Cellobiose and salicin were obtained from Sigma Chemical Company, and raffinose, cellobiose, maltose and lactose from Pfanstiehl Chemical Company. Glucose oxidase ("Glucostat") was obtained from Worthington Biochemical Corporation.

Preparation of homogenates

Roaches were starved for 48 hours prior to preparation and the tissues weighed and homogenized in Tris:HCl 0.005 *M*, pH 7.4 (2:1, v/w) with a Servall Omni-Mix at 0° C. for three minutes at 14,000 rpm. The resulting homogenate was squeezed through three thicknesses of cheesecloth, rehomogenized in a Tenbroek homogenizer, and the volume measured (Fraction I). An aliquot was subjected to centrifugation at 24,000 *g* at 0° C. for 30 minutes. The supernatant was poured through glass wool to remove the fatty layer and the supernatant volume measured (Fraction II).

Purification

An aliquot of Fraction II was subjected to ethanol precipitation at −7° C., similar to the method used by Friedman (1960). Absolute ethanol was added drop-wise with constant stirring until 30% v/v was obtained, and the mixture agitated for an additional 10 minutes. The mixture was centrifuged at 24,000 *g* (0° C.) for 30 minutes, the supernatant removed and measured, and sufficient ethanol added to that supernatant at −7° C. to bring the concentration to 40% (v/v). This process was continued step-wise in 10% increments until the ethanol percentage was 70%. Each of the precipitated fractions was taken up in 5.0 ml. Tris:HCl (0.005 *M*, pH 7.4) and dialyzed against two liters of that buffer at 5° C. for 18 hours. The precipitated material from the dialyzed fractions was removed by centrifugation and the supernatant stored at −20° C. until assay.

In some preparations (isolated gut tissue), the procedure was somewhat different. Fraction II of that preparation was subjected to 29% ethanol precipitation at −7° C., and subsequent precipitations were completed on the 29% ethanol supernatant with −20° C. acetone in a refrigerated bath at −7° C. Precipitated fractions of 30%, 40%, 60%, and 80% (v/v) acetone were collected, taken up in Tris: HCl buffer (0.005 *M*, pH 7.1), dialyzed against that buffer, centrifuged as above, and stored at −20° C.

Assay procedures

Enzyme activity was normally determined as follows: 0.1 to 0.3 ml. of the enzyme solution or diluted enzyme was added to 10 μmoles (0.5 ml.) substrate and 60 μmoles (0.6 ml.) buffer. Deviations from these conditions will be discussed in the text. The mixture was incubated at 34° C. for 15 minutes and the reaction was stopped by the addition of 1.0 ml. $Ba(OH)_2$ and 1.0 ml. $ZnSO_4$, according to the method of Somogyi (1952). After centrifugation, aliquots of supernatant were removed and glucose was determined with glucose oxidase (Keston, 1956) or by the Somogyi-Nelson method (Somogyi, *loc. cit.*). When nitrophenyl glycosides were used as substrates, the mixtures were made alkaline by the addition of 0.1 *N* $NaCO_3$ and read directly at 400 mμ. Standard glucose curves were established by adding the equivalent volume of the particular disaccharide or glycoside substrate to each individual concentration of glucose and the water blank. These tubes were treated with $Ba(OH)_2$ and $ZnSO_4$ in the same manner as the experimental tubes.

TABLE I

Enzymatic activity of Fraction II on various carbohydrates. Conditions: 10 μmoles substrate, 60 μmoles buffer (citrate pH 5.6), 0.1 ml. enzyme (whole animal preparation)

Substrate	μmoles/mg. protein/hour
Cellobiose	1.29
Sucrose	0.98
Trehalose	0.96
Lactose	0.84
Melibiose	0.68
Raffinose	0.68
Maltose	0.54
Starch	+
Glycogen	+
Cellulose (Alphacel)	−
Methyl cellulose	−

This system afforded internally compensated control for the contaminant carbohydrases in the glucose-oxidase preparation. On some occasions, the "Glucostat" was diluted with 0.20 *M* or 0.25 *M* Tris: HCl at pH 7.1 *in lieu* of water. This addition inhibited all carbohydrates at least 95%. The glucose-oxidase reaction was conducted with 4 ml. of the reagent at 34° C. for 30 minutes. The reaction was stopped and color developed by the addition of 0.05 ml. of 6 *N* HCl and the tubes read with a #42 filter in a Klett-Summerson colorimeter. Standard curves (corrected for substrates) obtained by this method gave slopes of 0.2 (±0.02) μgm. glucose/klett unit. Transferase activity was examined according to the method of Aronson (1952). Protein was estimated by the method of Lowry *et al.* (1951) with the Folin-Ciocalteu reagent, using crystalline Armour bovine plasma albumin as standard. It was found that Sigma bovine serum albumin gave the same standard curve as did the Armour bovine plasma product. Activity of the enzymes is expressed as μmoles substrate hydrolyzed/mg. protein/hour.

Paper chromatography and disc electrophoresis

Reaction mixtures were examined chromatographically, using the ascending technique on Whatman #1 paper and solvent system of ethyl acetate, pyridine,

TABLE II

Enzymatic activity of ethanolic fractionation of Fraction II. Conditions: Same as Table I

Ethanol fraction % v/v	μmoles/mg. protein/hour		
	Maltose	Cellobiose	Trehalose
30	0.80	1.0	0.5
40	0.80	1.25	0.90
50	0.80	2.20	1.50
60	2.50	4.00	1.40
70	6.10	0.50	0.20

methyl ethyl ketone, and water (5:4:4:1, v/v) or the descending method (Whatman #4 paper) with n-propanol, ethyl acetate, and water (7:1:2, v/v) (Barr and Bull, 1953). A modified method of the $AgNO_3$: NaOH of Trevelyan *et al.* (1950) was used to determine the carbohydrates. The chromatograms were sprayed with the acetone : $AgNO_3$ solution *in lieu* of dipping as suggested by the

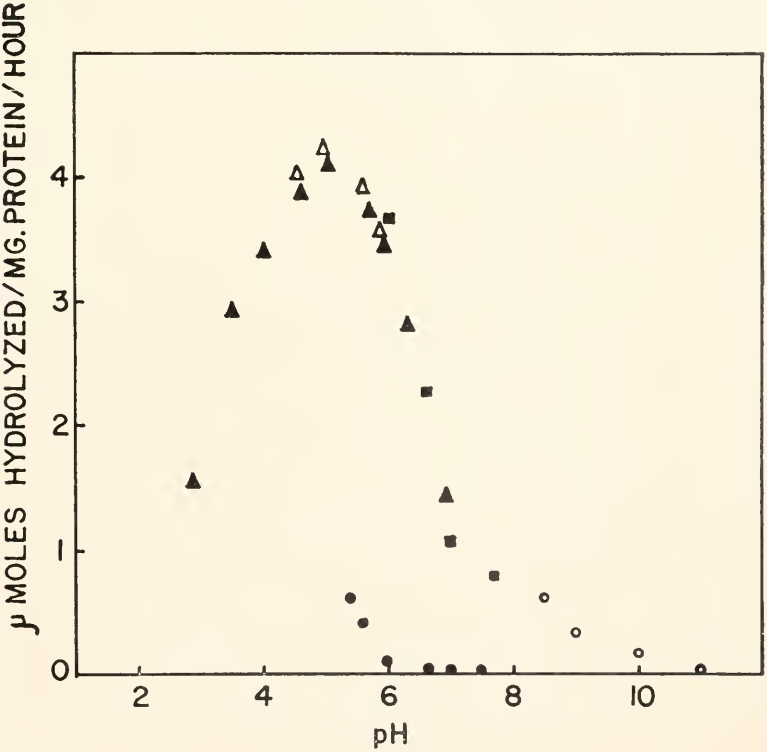


FIGURE 1. The pH optimum of *Blaberus* β-glucosidase. Conditions: 10 μmoles cellobiose, 0.1 ml. enzyme, 60 μmoles buffer. ▲-▲-citrate phosphate, ●-●-Tris : maleate, ■-■-phosphate (K^+), △-△-phthalate(Na^+), ○-○-glycine (Na^+).

authors. Chromatograms were also examined under ultraviolet light, and many of the aryl-aglycone moieties of the glucosides were visualized.

Protein fractions were examined by disc electrophoresis (Reisfeld *et al.*, 1962) on acrylamide gel incorporating Tris: glycine buffer, pH 8.3 with a potential of 200 volts. The protein bands were localized with amido black.

RESULTS

Total Animal Preparation

Hydrolysis of carbohydrates

Assay of the supernatant of total animal homogenates (Fraction II) affords some idea of the number of carbohydrases present in *Blaberus craniifer* (Table I). In addition to those substrates shown, glycogen and starch were hydrolyzed; however, cellulose and methyl cellulose were not hydrolyzed. Precipitation of Fraction II with ethanol at −7° C. partially separated and increased the specific activity of the preparations (Table II). The α-glucosidase active against maltose precipitated largely in the 70% ethanol fraction, while the β-glucosidase activity precipitated out in the 50% and 60% fractions. Trehalase was also equally distributed in the 50% and 60% fractions.

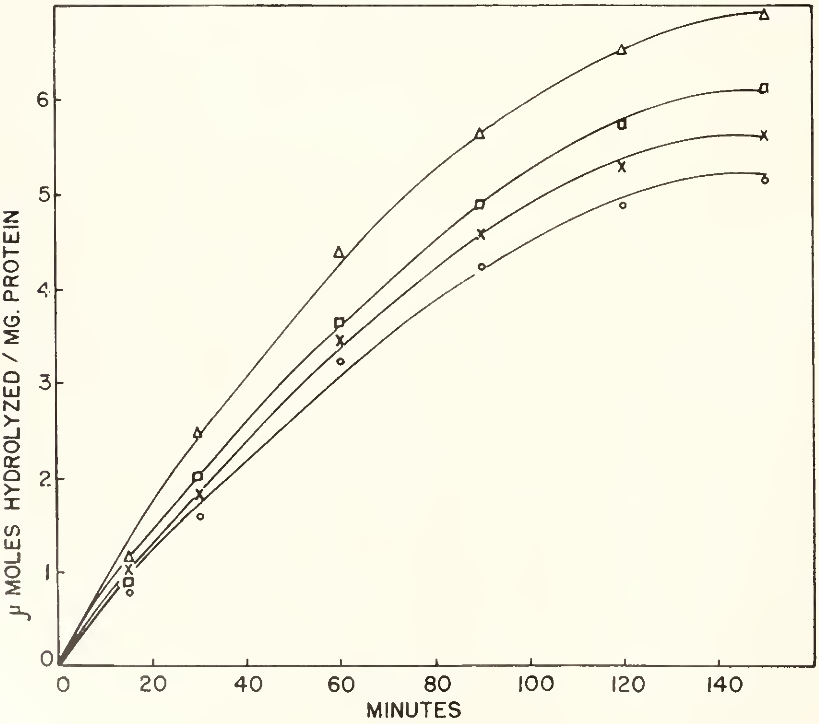


FIGURE 2. Effect of pH and increasing time on enzymatic activity. Phthalate (Na$^+$) buffers △-△ pH 5.0, □-□ pH 4.4, ×-× pH 5.6, ○-○ pH 5.8. Conditions: 10 μmoles cellobiose, 60 μmoles buffer, 0.1 ml. enzyme (40% to 60% ethanol fraction, whole animal).

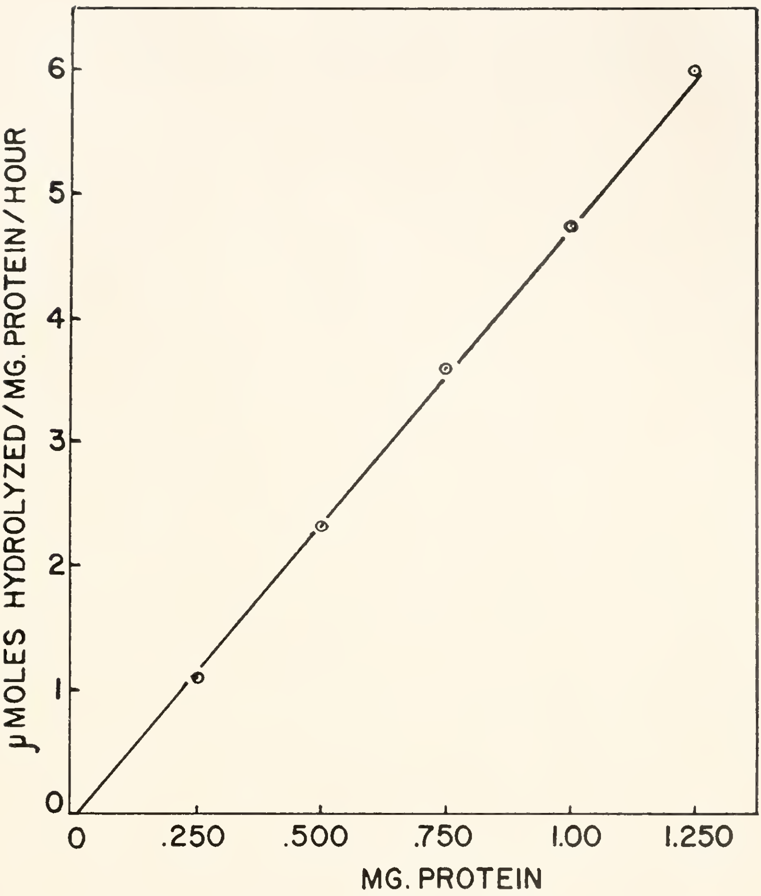


FIGURE 3. Enzymatic activity *vs.* protein concentration. Assay conditions: 10 μmoles cellobiose, 60 μmoles buffer (phthalate pH 5.0), 0.1 ml. enzyme (40% to 60% ethanol fraction, whole animal).

Effect of pH

In examining the hydrolysis of cellobiose, five buffers were used which covered a range from pH 2.9 to 11.0 (Fig. 1). The curve represents the average of four determinations at each point. At the concentration used (4.29×10^{-2} M), the Tris:maleate buffer inhibited cellobiose hydrolysis some 85%, while trehalose hydrolysis was inhibited only 25% under the same conditions. This is based on 100% activity in pH 5.0 phthalate buffer with which the highest activity was observed.

Effect of time and enzyme concentration

Figure 2 shows the effect of increasing time on cellobiose hydrolysis using four phthalate buffers, and is based on the average of triplicate observations. This

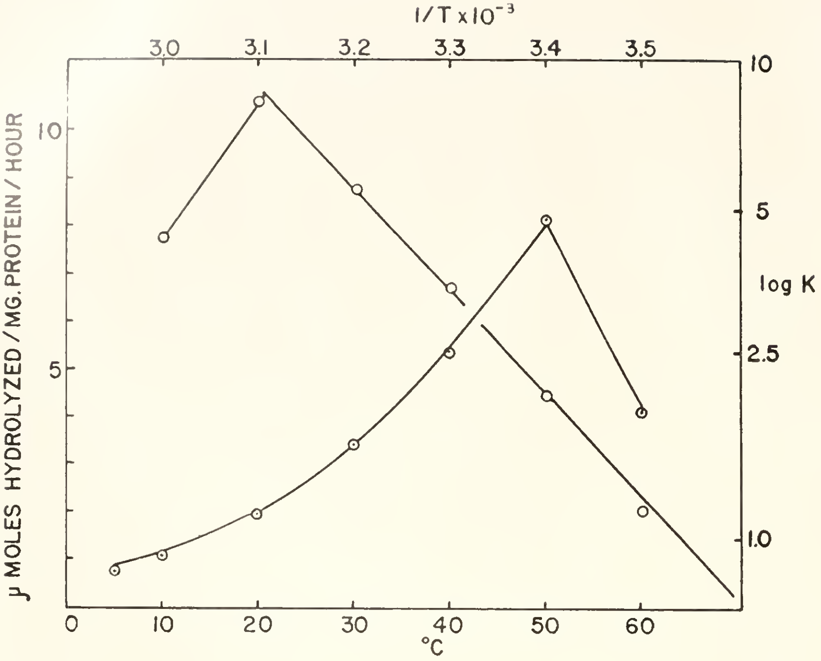


FIGURE 4. Dual plot of enzymatic activity *vs.* increasing temperature (⊙-⊙) and Arrhenius plot ($1/T$ *vs.* log K) (○-○). Conditions: 10 μmoles cellobiose, 60 μmoles buffer (phthalate pH 5.0), 0.1 ml. enzyme (40% to 60% ethanol fraction, whole animal).

TABLE III

Effect of metal ions on enzymatic activity. Conditions: 10 μmoles cellobiose, 60 μmoles buffer (phthalate pH 5.0), 0.01 ml. inhibitor, 0.1 ml. enzyme (40% to 60% ethanol fraction, whole animal)

Inhibitor ion	Final concentration (molar)	% Inhibition
None	--	0
Ag^+	3.00×10^{-5}	100
Ag^+	8.26×10^{-4}	100
Hg^+	8.26×10^{-4}	72
Hg^{++}	8.26×10^{-4}	65
Cu^{++}	8.26×10^{-4}	60
Pb^{++}	8.26×10^{-6}	50
Fe^{++}	8.26×10^{-4}	40
Co^{++}	8.26×10^{-4}	18
Zn^{++}	8.26×10^{-4}	16
Fe^{+++}	8.26×10^{-4}	14
Sn^{++}	8.26×10^{-4}	9
Al^{+++}	8.26×10^{-5}	8
Mn^{++}	8.26×10^{-4}	4
Mg^{++}	8.26×10^{-4}	4
Cu^+	8.26×10^{-4}	2

figure indicates that the hydrolysis is inhibited more on the alkaline side of the peak than on the acid side. Enzymatic activity was linear as a function of protein concentration (Fig. 3), and no inhibition of activity was noted throughout the range described.

Energy of activation

The energy of activation for cellobiose hydrolysis was determined from the linear portion (5° C. to 50° C.) of an Arrhenius plot (log K *vs.* $1/T$) where the slope represents $-E/2.303\ R$. Figure 4 represents the effects of increasing temperature *vs.* activity, as well as the Arrhenius plot. From these data based on triplicate observations, the energy of activation was estimated to be 9400 calories/mole. It was further determined that 50% of the hydrolytic activity against cellobiose was lost following incubation for one hour at 50° C.

Effect of metals and salts

The inhibitory nature of Tris buffers on cellobiose hydrolysis has been mentioned previously. This is also inhibitory for a number of other glucosidases (Friedman, 1960; Mahler, 1961; White and Subers, 1961; Blecher and Glassman, 1962). Table III lists 16 metal ions and their inhibition activity, using pH 5.0 phthalate buffer as 100%. Enzyme activity was reduced by the specific sulfhydryl inhibitors, parachloromercuribenzoic acid (PCMB), phenylmercuriacetate (PMA), iodoacetate, and iodacetamide; and cysteine produced a partial reversal of these inhibitions. Iodoacetate and iodoacetamide at $2 \times 10^{-4}\ M$ completely inhibited the enzyme, and the reversal by $4 \times 10^{-3}\ M$ cysteine was significant (Table IV). PCMB and PMA were used at lower concentrations, and significant reversal by

TABLE IV

Effect of sulfhydryl inhibitors. Conditions: 10 μmoles cellobiose, 60 μmoles buffer (phthalate pH 5.0), 0.1 ml. enzyme (Fraction II, whole animal)

Inhibitor	Cysteine (Molar)	Concentration inhibitor (Molar)	% Inhibition
None	0	—	0
	4×10^{-3}		0
PCMB	0	2×10^{-6}	40
	4×10^{-3}	2×10^{-6}	9
	0	4×10^{-5}	62
	4×10^{-3}	4×10^{-5}	13
PMA	0	2×10^{-5}	56
	4×10^{-3}	2×10^{-5}	11
Iodoacetate	0	2×10^{-4}	100
	4×10^{-3}	2×10^{-4}	83
Iodoacetamide	0	2×10^{-4}	96
	4×10^{-3}	2×10^{-4}	79

cysteine was also observed. The effect of monovalent cations on enzymatic activity was examined, using the chloride form at a final concentration of 1.67×10^{-1} *M*. The following relationship existed:

$$K^+ > Na^+ > Rb^+ > Cs > Li$$

In triplicate experiments, it was found that the overall activation of potassium was 10%, while that of lithium was less than 2%. The other cations were displaced between those points. In this series of experiments, the enhancer effect of toluene (Veibel, 1937–38) was also examined, and no increase in activity was observed.

Localization of enzyme

In assays of the total animal, isolated gut, and carcass, it was found in eight determinations that 89–95% of the β-glucosidase activity was in the gut. When the gut was divided into three portions (*i.e.*, fore-, mid-, and hindgut), it was observed that 96% of the total gut activity resided in the ceca complex. Histochemical localization of the enzyme was determined according to Pearse (1960), using 6-bromo-2-naphthol-β-glucoside as the substrate, and after coupling with fast blue B. The major enzymatic activity was found in the ceca and a small amount in the midgut tissue. The only localized area of activity in the carcass was found in the epidermis; however, the colleterial glands were not examined. The right colleterial gland has been shown in other roach species to have a β-glucosidase, and the left gland contained aryl-β-glucosides. The concomitant mixing of the secretions of these two glands resulted in the liberation of the aglycone, which was oxidized by a phenolase to a quinone. The result was a general darkening of the oötheca (Brunet and Kent, 1955; Stay and Roth, 1962).

TABLE V

Ethanol-acetone fractionation of gut tissue

Fraction	Volume (ml.)	Mg. protein (ml.)	Total protein (mg.)	μmoles Mg. protein/hour	Total activity	% Recovery supernatant
Homogenate	44	29.8	1311.1	4.68	6135.9	—
Supernatant	34	20.1	683.4	6.52	4455.8	100
29% Ethanol	15.5	26.6	413.0	1.69	698.0	15.7
20% Acetone	4.2	.96	4.0	3.85	15.4	0.3
40% Acetone	4.4	1.65	7.3	3.71	27.1	0.6
60% Acetone	7.0	2.34	16.4	173.21	2840.6	63.8
80% Acetone	2.0	1.20	2.4	1.5	3.6	0.1

Isolated Gut Preparation

The enzyme used in the following series was the 60% acetone fraction, prepared as described in the methods section. Table V summarizes this purification and gives the specific activities of the fractions against cellobiose. The pH optimum and the effect of metal salts were the same as obtained from the total animal preparation.

Specificity of the fraction

The specific activities of the 60% acetone fraction against 20 substrates are listed in Table VI. Five of the ten β-linked glucosides tested were hydrolyzed by this preparation. Heat treatment at 50° C. for one hour decreased the hydrolytic properties toward all of those five glucosides approximately 50%. Lactose and ONPGal were hydrolyzed, as well as trehalose and maltose. Examination of this enzyme preparation by disc electrophoresis showed two distinct small bands and a larger band which contained two components.

TABLE VI

Specific activities of 60% acetone fraction at pH 5.0 against various substrates. Conditions: 20 μmoles substrate, 60 μmoles buffer (phthalate pH 5.0), 0.1 ml. enzyme (60% acetone, animal gut)

β-glucosides	μmoles/mg. protein/hour
Cellobiose	173.2
Phenyl-β-D-glucoside	84.3
p-nitrophenyl-β-D-glucoside	44.1
Salicin	34.6
Arbutin	22.7
Gentiobiose	—
Amygdalin	—
β-methyl-D-glucoside	—
Cellulose (alpha-cel)	—
Methyl cellulose	—
Other carbohydrates	**μmoles/mg. protein/hour**
o-nitrophenyl-β-D-galactopyranoside	41.2
Lactose	33.1
Trehalose	28.1
Maltose	15.8
β-melibiose	—
Melibiose	—
Sucrose	—
Raffinose	—
Starch	—
Glycogen	—

Transferase activity

No transferase activity was observed when methanol, ethanol, glucose, hydroquinone and p-nitrophenol were used as acceptors and cellobiose as the donor. Mixtures fortified with adenosine triphosphate (ATP) and/or uridine-diphosphoglucose (UDPG) were negative for transferase and/or uridine-diphosphate-glucose glycosyltransferase activity. Reaction mixtures were examined chromatographically, and only increasing amounts of glucose and decreasing amounts of cellobiose were observed in reaction mixtures incubated 0.25, 0.5, 1, 3, 6, 18, 24, and 120 hours. Dutton (1962) has reported that intact and homogenized cecum and fat body of *Periplaneta* bring about the conjugation of o-aminophenol only in the presence of UDPG.

Effect of substrate concentration and inhibition

The effect of cellobiose concentration *vs.* enzymatic activity was examined using nine substrate concentrations over a 100-fold range. The K_m was estimated to be 5.63×10^{-4} M and the V_{max} 1.89×10^2 μmoles/mg. protein hour from the double

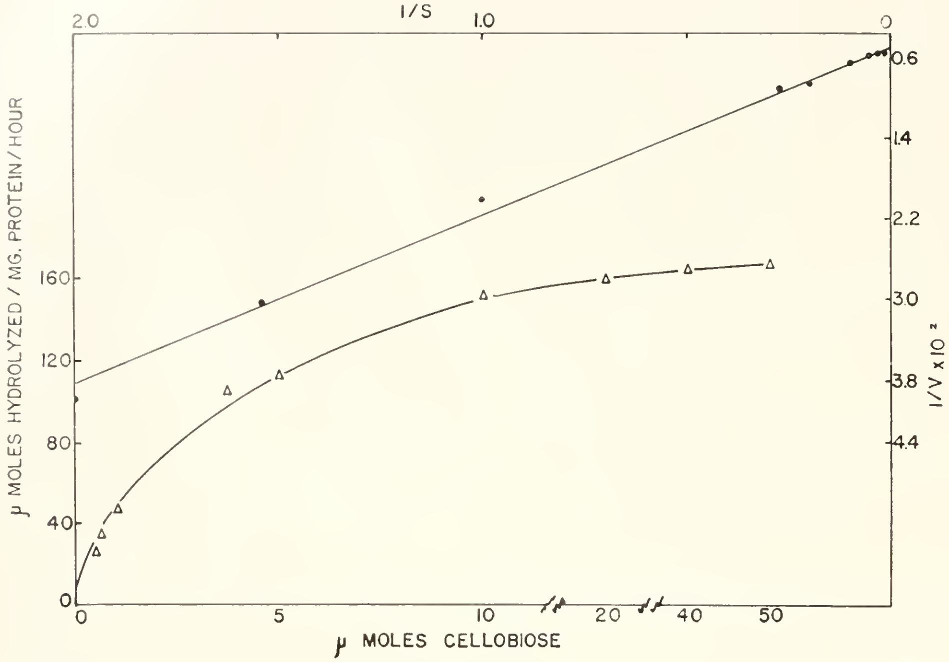


FIGURE 5. Substrate concentration plot: (1) △-△ specific activity *vs.* concentration of cellobiose; (2) ●-● $1/v$ *vs.* $1/s$. Conditions: 60 μmoles buffer (phthalate pH 5.0), HOH + substrate to equal 1.7 ml., 0.1 ml. enzyme (60% acetone fraction, animal gut).

TABLE VII

K_m and K_{max} values against various glucosides. Conditions: 20 μmoles substrate, 60 μmoles buffer (phthalate pH 5.0), 0.1 ml. enzyme (60% acetone fraction, animal gut)

Substrate	Inhibitor	$K_m \times 10^3$	Apparent $K_m \times 10^3$	V_{max}	$Ki \times 10^3$
p-nitrophenol β-D-glucoside	—	16.56		62.5	—
	Lactose 2.5×10^{-2}		56.17	55.6	9.0
	Cellobiose 2.5×10^{-2} M		126.40	58.8	3.0
	Glucose 1.25×10^{-2} M		65.07	58.1	4.0
o-nitrophenol-β-D-galactopyranoside	—	389.1		76.9	—
	Lactose 2.5×10^{-2} M		426.8	77.9	303.0
	Cellobiose 2.5×10^{-2} M		847.8	66.7	15.0
	Glucose 1.25×10^{-2} M		410.2	83.3	136.0
Phenyl-β-D-glucoside		23.2		14.3	
Arbutin		1.2		14.7	
Salicin		0.621		17.2	
Cellobiose		0.563		188.6	

reciprocal plot (Fig. 5). The K_m and V_{max} values for other β-glucosides and ONPGal are presented in Table VII. Also given in that figure are the results obtained when PNPG and ONPGal were used as substrates and lactose, cellobiose and glucose as inhibitors.

Further purification of 60% acetone fraction

A portion of the 60% acetone fraction was dialyzed against 0.005 *M* Tris: maleate, pH 8.4, and placed on a 1 × 15 cm. DEAE cellulose column prepared according to Peterson and Sober (1956) and a gradient elution effected with a two-container closed system under 1.5 psi nitrogen gas at 5° C. The mixing chamber contained 200 ml. Tris:maleate 0.005 *M*, pH 8.4, and the flask delivering to the mixing chamber contained 700 ml. of the same buffer at pH 4.6. After the above elution was complete, the mixing chamber was charged with 500 ml. Tris: maleate 0.005 *M*, pH 4.6, and 200 ml. 0.5 *N* NaCl was added to the delivery flask. When 100 ml. ± 25 ml. remained in the mixing chamber, 200 ml. of 2.0 *N* NaCl were added to the delivery flask and elution continued. Fifteen-milliliter-samples were collected throughout the elution. The profile of protein eluted from the column is shown in Figure 6. The pH gradient is presented, as well as the Cl^- concentration as meq/l. Chloride was determined with a Cotlove chloridometer and pH with a Beckman probe-type electrode. Beta-glucosidase activity is indicated by the hatched area. Beta-glucosidase activity and a trace of β-galactosidase

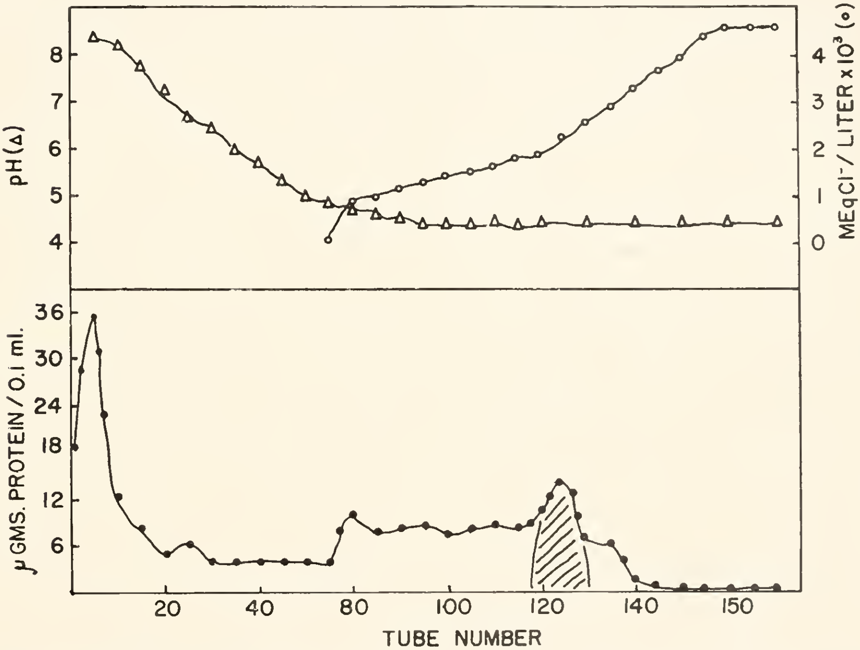


FIGURE 6. Elution patterns from DEAE cellulose column. The pH curve is designated by: Δ-Δ, Cl (meq/l.) by ○-○; and protein by ●-●, shaded area = enzymes.

activity were observed in the peak. Lyophilization of the protein from the enzyme peak after preliminary assays destroyed about 96% of the activity. Alpha-glucosidase and trehalase activities were not in the indicated peak of enzyme activity.

DISCUSSION

Protein preparations from *Blaberus* hydrolyze numerous carbohydrates, and these enzymatic activities may be partially separated by ethanol precipitation at −7° C. (Figs. 1 and 2). The major source of hydrolytic enzymes is the intestine; however, the extra-intestinal tissues possess at least trehalase and a β-glucosidase. Maximum β-glucosidase activity is found at pH 5.0 (Fig. 3). The effect of increasing time on the hydrolysis of cellobiose in four phthalate buffers shows that maximum hydrolysis in 150 minutes occurs at pH 5.0; 89% of that activity was observed at pH 4.4, 81% at pH 5.6, and 74% at pH 5.8 (Fig. 4). The rate of hydrolysis was little affected by the addition of Na^+, K^+, Li^+, Rb^+, Cs^+ and Cu^+; however, Ag^+ and Hg^+ had a pronounced inhibitory effect. Among the divalent ions (Fig. 7), Sn^{++}, Mn^{++} and Mg^{++} were without appreciable effect, while Hg^{++}, Cu^{++}, Pb^{++}, Co^{++}, Zn^{++} and Fe^{++} inhibited hydrolysis in excess of 14%. Of the trivalent metals tested, Fe^{+++} was more effective than Al^{+++}. Similar results have been reported for β-glucosidases of almond (Veibel, 1951); and for β-glucosidase (Duerksen and Halvorson, 1958), α-glucosidase (Halvorson and Ellias, 1958), and invertase of yeast (Myrback, 1957). The effect of these metal ions suggested the presence of sulfhydryl groups (−SH); and the inhibitory effect of −SH inhibitors was examined, as well as the reversal effect of cysteine. Iodoacetate, iodoacetamide, PCMB and PMA all provided inhibition of hydrolysis of cellobiose; the inhibition was partially reversed by the addition of 4×10^{-3} *M* cysteine (Fig. 8). No studies were undertaken which would elucidate the involvement of these groups in hydrolytic activity, as has been shown by competitive substrate protection experiments with yeast β-glucosidase (Duerksen and Halvorson, 1958).

By treatment of the ethanolic supernatant fraction (29% v/v) of the roach gut preparation with increasing percentages of acetone (v/v), the specific activity was increased some 37-fold (Fig. 9). The 60% acetone fraction from this purification was tested against a number of carbohydrates (Fig. 10). Of the β-linked carbohydrates examined, cellobiose, phenyl-β-D-glucoside, PNPG, salicin and arbutin were hydrolyzed. Amygdalin, gentiobiose, β-methyl-D-glucoside, cellulose and methyl cellulose were not hydrolyzed. These data suggest that the $\beta 1 \rightarrow 6$ linkage of gentiobiose and the glycone of amygdalin, which is also gentiobiose, cannot be hydrolyzed by this enzyme. The fact that cellulose cannot be hydrolyzed indicates that no exoglucosidase activity is present. Also, methyl cellulose and β-methyl-D-glucoside are not hydrolyzed; and this suggests that there is some specificity toward the aglycone moiety of the glucoside. Similar results have been discussed by Jermyn (1961).

It is of interest that the partial inactivation of the enzyme by heat results in the proportional loss (*ca.* 50%) of activity toward all of the five β-glucosides hydrolyzed. This favors the notion that each of these glucosides is hydrolyzed by a common site, as reported by Weidenhagen (1932). Under similar treatment, lactose, maltose and trehalose hydrolysis was decreased 40%, 62% and 59%, respectively.

The 60% acetone fraction was further examined by using aryl substrates with cellobiose, glucose, and lactose as inhibitors. Cellobiose and glucose were competitive inhibitors of PNPG, and lactose was competitive against ONPGal. On comparing the K_i values (Fig. 12) which were determined from the calculated slopes, it is obvious that two enzymes or two active centers are present, one for β-glucosides and another for β-galactosides. This is not consistent with the notion that these two classes of compounds are hydrolyzed at the same enzyme site, as reported for almond emulsion by Heyworth and Walker (1961).

The author wishes to thank Mrs. Marillyn Gragg and Mr. Robert McKitchie for technical assistance. Thanks are also extended to Dr. C. P. Read for his comments and criticisms and to Dr. R. C. Sanborn for the loan of equipment.

SUMMARY

1. The localization, properties and specificity of a β-glucosidase from *Blaberus craniifer* have been investigated.

2. The enzyme is localized mainly in the cecal complex of the alimentary canal.

3. Evidence is presented which suggests that all the β-glucosides hydrolyzed are hydrolyzed by the same enzyme and that the β-galactosides are hydrolyzed by another protein or series of active sites.

4. The data also suggest that some specificity of the enzyme is directed toward the aglycone moiety of the β-glucoside.

5. Based on inhibition of the enzyme by heavy metals and specific —SH inhibitors and the reversal of —SH inhibition by cysteine, it is suggested the enzyme is an —SH enzyme.

6. No effects of toluene were noted and the effects of monovalent cations were less than 10%.

7. The K_m, V_{max} and K_i values for various glucosides are presented.

LITERATURE CITED

Aronson, M., 1952. Transgalactosidation during lactose hydrolysis. *Arch. Biochem. Biophys.*, **39**: 370–378.

Barr, S., and J. P. Bull, 1953. Salt interference in sugar chromatography of urine. *Nature*, **172**: 414–415.

Blecher, M., and A. B. Glassman, 1962. Determination of glucose in the presence of sucrose using glucose oxidase; effect of pH on absorption spectrum of oxidized o-dianisidine. *Analyt. Biochem.*, **3**: 343–351.

Brunet, P. C., and P. W. Kent, 1955. Mechanism of sclerotin formation—the participation of a beta-glucosidase. *Nature*, **175**: 819–820.

Duerksen, J. D., and H. Halvorson, 1958. Purification and properties of an inducible β-glucosidase of yeast. *J. Biol. Chem.*, **233**: 1113–1120.

Dutton, G. J., 1962. The mechanism of o-aminophenyl glucoside formation in *Periplaneta americana*. *J. Comp. Biochem. Physiol.*, **7**: 39–46.

Ehrhardt, P., and G. Voss, 1962. Beitrag zum Wirkungsspektrum Kohlenhydratspaltender Fermente und ihre Verteilung im Verdauungstrakt der Schaben *Blaberus discoidalis* Sv. und *Leucophaea madeirae* [sic] F. (Blattoidea). *J. Ins. Physiol.*, **8**: 165–174.

Evans, W. A. L., 1956. Studies on the digestive enzymes of the blowfly *Calliphora erythrocephala*. I. The carbohydrases. *Exp. Parasitol.*, **5**: 191–206.

Evans, W. A. L., and D. W. Payne, 1960. Carbohydrases in the alimentary tract of the locust *Schistocerca gregaria* (Forsk.). *Biochem. J.*, **76**: 50.

Fraenkel, G., 1940. Utilization and digestion of carbohydrate by the adult blowfly *Calliphora erythrocephala*. *J. Exp. Biol.*, **17**: 18–29.

Friedman, S., 1960. The purification and properties of trehalase from *Phormia regina* Meig. *Arch. Biochem. Biophys.*, **87**: 252–258.

Halvorson, H., and L. Ellias, 1958. The purification and properties of an α-glucosidase from *Saccharomyces italicus* Y 1225. *Biochim. Biophys. Acta*, **30**: 28–40.

Heyworth, R., and P. G. Walker, 1961. Identity of β-glucosidase and β-galactosidase in sweet-almond emulsin. *Biochem. J.*, **80**: 44.

Ito, T., and M. Tanaka, 1959. Beta-glucosidase of the mid gut of the silkworm *Bombyx mori*. *Biol. Bull.*, **116**: 95–105.

Jermyn, M. A., 1961. Glycosidases. *Revs. Pure and Applied Chem.*, **11**: 92–116.

Keston, A. S., 1956. Specific colorimetric enzymatic reagents for glucose. *Abstracts, A.C.S., Div. Biol. Chem.*, April: 31c.

Koike, H., 1954. Studies on carbohydrases of insects. I. Distribution of carbohydrases in several insects. *Zool. Mag. (Tokyo)*, **63**: 228–234.

Lasker, R., and A. C. Giese, 1956. Cellulose digestion by the silverfish *Ctenolepisma lineata*. *J. Exp. Biol.*, **33**: 542–553.

Lipke, H., and G. Fraenkel, 1956. Insect nutrition. *Ann. Rev. Entomol.*, **1**: 17–44.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

Mahler, H. R., 1961. The use of amine buffers in studies with enzymes. *Ann. N. Y. Acad. Sci.*, **92**: 426–439.

Myrback, K., 1957. Inhibition of yeast invertase (saccharase) by metal ions. V. Inhibition by mercury compounds. *Arkiv. Kemi.*, **11**: 471–479.

Newcomer, W. S., 1952. The occurrence of beta-glucosidase in the digestive juice of *Porcellio* and *Armadillidium*. *Anat. Rec.*, **113**: 536.

Newcomer, W. S., 1954. The occurrence of β-glucosidase in the digestive juice of the cockroach, *Periplaneta americana* L. *J. Cell. Comp. Physiol.*, **43**: 79–86.

Newcomer, W. S., 1956. Digestive carbohydrases of the wood louse, *Porcellio*. *Physiol. Zoöl.*, **29**: 157–162.

Pearse, A. G. E., 1960. Histochemistry. Little, Brown and Co., Boston. 998 pp.

Peterson, E. A., and H. A. Sober, 1956. Chromatography of proteins. I. Cellulose ion exchange adsorbents. *J. Amer. Chem. Soc.*, **78**: 751–775.

Powning, R. F., and H. Irzykiewicz, 1962. β-glucosidase in the cockroach (*Periplaneta americana*) and in the puffball (*Lycoperdon perlatum*). *Comp. Biochem. Physiol.*, **7**: 103–115.

Reisfeld, R. A., V. J. Lewis and D. E. Williams, 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature*, **195**: 181–183.

Robinson, D., 1956. The fluorometric determination of β-glucosidase: its occurrence in the tissues of animals, including insects. *Biochem. J.*, **63**: 39–44.

Somogyi, M., 1952. Notes on sugar determination. *J. Biol. Chem.*, **195**: 19–23.

Stay, B., and L. M. Roth, 1962. The colleterial glands of cockroaches. *Ann. Entomol. Soc. Amer.*, **55**: 124–130.

Trevelyan, W. E., D. P. Procter and J. S. Harrison, 1950. Determination of sugars on paper chromatograms. *Nature*, **166**: 444–445.

Veibel, S., 1937–38. Über den Tolnol-Effekt bei der fermentativen Hydrolyse von β-d-glucosiden. *Enzymologia*, **2**: 367–372.

Veibel, S., 1951. *In:* J. B. Sumner and K. Myrbäck (Editors), The Enzymes, Vol. I, Acad. Press, Inc., New York. pp. 583.

Weidenhagen, R., 1932. Spezifität und Wirkungmechanismus der Carbohydrasen. *Ergebn. Enzymforsch.*, **1**: 168–208.

White, J. W., Jr., and M. H. Subers, 1961. A glucose oxidase reagent for maltose assay. *Anal. Biochem.*, **2**: 380–384.

NOSEMA AS A SOURCE OF JUVENILE HORMONE IN PARASITIZED INSECTS[1]

FRANK M. FISHER, JR.[2] AND RICHARD C. SANBORN

Department of Biological Sciences, Purdue University, Lafayette, Indiana

The effects resulting from parasitic invasion of insects with respect to abnormalities of the host's morphology and development have been known for many years. Yet the precise mechanism by which these often singular changes are caused remain for the most part obscure, due largely to a lack of detailed knowledge of the physiology of the host-parasite relationship.

The endocrinological control of development and metamorphosis in insects is now well oriented (see reviews by Bodenstein, 1954; Scharrer, 1955; Van der Kloot, 1960, 1962; Karlson, 1963; Schneiderman and Gilbert, 1963) and it appears that some basic questions may be asked concerning insect-parasite relationships and, in particular, those associations where there appears to be a hormonal involvement.

Three hormonal factors are involved in the control of growth and morphogenesis of insects. The first is a neurosecretory product of certain specialized cells in the pars intercerebralis of the brain, which initiates secretion of the second hormone, ecdysone, by the prothoracic glands. Ecdysone, the growth and differentiation hormone, acts upon the cells of the insect to induce growth and differentiation to the adult state unless the third hormone, juvenile hormone (neotenin), is present. Ecdysone and juvenile hormone act in concert to retard metamorphosis so that the animal grows but remains immature.

Several investigators have presented evidence for either the accelerated or retarded development due to parasitism of insect hosts. Acceleration of host deve'opment is well established. Alston (1920) reported that *Calliphora* (Diptera) when attacked by *Alysia* (Braconidae) pupated immediately. Salt (1932) reported a similar example with *Lucilia* (Diptera) serving as the host. He also stated that starved or half-grown *Calliphora* larvae, which would normally fail to pupate, pupated immediately following a sting by the parasite. Varley and Butler (1933) found that *Polemon*, a braconid, upon infecting *Lipara* (Diptera) accelerated its development by six months; but the break in diapause for this host is apparently due to an effect of the parasite within the host, since the termination of diapause occurred after some development of the parasite. These authors also stated that when *Urophora*, a trypetid fly, is attacked by *Eurytoma* (Chalcididae) pupation of the host occurred eight months earlier than it does in non-parasitized controls. More recently Schneider (1950, 1951) observed that *Diplazon* (Ichneumonidae) when parasitizing the syrphid, *Epistrophe*, begins to feed immediately, but has little effect upon the host until the host has been diapausing about one month. The cool weather of autumn apparently activates the parasite larvae which then molt. The molting hormone secreted by the endocrine glands of the parasite appears to diffuse through the parasite's body wall, causing the host to form a premature puparium. Following the induced pupation the parasite histolyzes the contents of the host puparium and itself enters diapause as a mature fifth instar larva.

---

[1] Aided by grants from the Purdue Research Foundation and from the National Science Foundation (G-11234).

[2] Present address: Department of Biology, Rice University, Houston 1, Texas.

Schneider transversely ligated a diapausing *Epistrophe* larva and injected a larva of *Diplazon* into the posterior chamber of the ligatured host. The posterior end of the ligatured host formed a puparium while the portion anterior to the ligature remained larval. He concluded that pupation of the host is not controlled by its own endocrine centers since they are all anterior to the ligature.

In contrast to the above examples of accelerated development, Strickland (1911) reported that the development of the histoblasts of *Simulium* (Diptera) larvae infected with *Mermis* (Nematoda) and *Glugea* (Protozoa) is inhibited to a point where pupation becomes impossible. He also reported that infected *Simulium* larvae attain three times normal size.

Keilin and Robinson (1933) reported the effects of a nematode, *Aproctonema*, parasitic in the body cavity of *Sciara* (Diptera) which delays metamorphosis of that host. Halcrow (1954) showed that the ciliate, *Glaucoma*, retards the development of *Anopheles* (Diptera). Many of the infected pupal hosts possess larval heads attached to a misshapen cephalothorax. Larval setae were admixed with pupal setae and pupal "paddles" (*sic*) were malformed. None of the parasitized hosts gave rise to adult forms.

Finlayson and Walters (1957) described the abnormal metamorphosis of saturniid moths infected with a sporozoan, *Nosema*. Patches of pupal cuticle were scattered amidst normal adult cuticle over the underlying sporozoan infections in the fat body. The authors concluded that the sporozoan either stimulates the host to produce excess juvenile hormone or the *Nosema* itself produces a substance with juvenile hormone activity. Since one adult with pupal characteristics emerged from an allatectomized pupa infected with the sporozoan, it would appear that the parasite is contributing a substance with juvenile hormone activity; but on the sole basis of one experimental animal it is difficult to accept this proposal.

More recently, West (1960) and Fisher and Sanborn (1962) reported that *Tribolium* (Coleoptera) larvae infected with a species of *Nosema* attained larger size than unparasitized controls, that few infected larvae reached the pupal stage and those which did pupate usually did not develop further.

Considering especially the latter observations and with knowledge of the earlier reports, it becomes clear that the microsporidian, *Nosema*, might well afford unequaled opportunity to investigate the endocrinological implications of host-parasite relationships. Is the parasite, *Nosema*, stimulating the insect hosts to produce more juvenile hormone or is that parasite producing a substance with juvenile hormone activity? It appears to us that the influence of this sporozoan parasite on its insect host may well form a model system to study such a host-parasite integration.

This paper reports experimental demonstrations of the effect of such a microsporidian insect parasite upon the normal hormonal regulation of its host's growth and metamorphosis.

MATERIALS AND METHODS

Cultural methods for the insects and *Nosema* used in these experiments have been described previously (Fisher and Sanborn, 1962).

Allatectomy of cockroaches

The techniques of allatectomizing Blattaria were based upon the methods of Bodenstein (1953) and of Scharrer (1946). Fifth or sixth stage nymphs were used in all experiments involving this operation.

The anterior edge of the pronotum of a newly molted, anesthetized nymph was removed to expose the head and cervical region. The cockroach was then transferred to an operating dish formed from lucite. Carbon dioxide was administered through an inlet into a space below a false bottom. A circular wax block was affixed to the upper surface of the false bottom, carbon dioxide passed through the false bottom and wax block, flowing over the ventral surface of the animal.

The cockroach was pinned to the wax block by placing a #1 insect pin across the neck near its union with the thorax. One end of the pin was thrust into the wax block and the other held down by a second pin bent into the shape of a "Z." Two # 1 pins were then placed through the lateral edges of the pronotum and the cockroach pulled posteriorly to stretch its neck. The pronotal pins were then secured to the wax block (Fig. 1, a).

The primary incision was made between the epicranium and cervical sclerites, which were then grasped with forceps, pulled posteriorly, and placed under the trimmed edge of the pronotum. This technique retracted the flap of cervical skin from the operation area. When excess hemolymph was blotted from the wound, the dorsal cervical tracheae and cervical musculature were revealed (Fig. 1, b). The dorsal tracheae were retraced laterally, and the opalescent blue corpora cardiaca were exposed, lying dorsal to the esophagus (Fig. 1, c). Tracing posteriorly along the corpora cardiaca, the spherical, translucent corpora allata were visualized dorsal or slightly lateral to the esophagus.

The posterior portion of each corpus cardiacum was grasped with forceps while another pair was used to free the corpus allatum of nervous connectives, tracheae and connective tissue (Fig. 1, d). To excise the corpus allatum the distal end of the corpus cardiacum was grasped with forceps between the corpus allatum and pulled posteriorly until the corpus allatum was free from the corpus cardiacum (Fig. 1, e). The excised corpora allata were examined under 30 to 100 × magnification to verify the intactness of each gland. If either gland was not complete the cockroach was discarded since it was nearly impossible to remove fragments of corpus allatum tissue. Finally, the cervical tracheae were returned to their normal position, the flap of cervical skin replaced and the wound was closed with a 1:1 mixture of petroleum jelly and beeswax (Fig. 1, f and g). Surgical and/or histological examinations were conducted at the termination of all experiments to ascertain the absence of corpora allata tissue.

Allatectomy of lepidopteran larvae

Galleria mellonella and *Bombyx mori* were also allatectomized during this study. Techniques for these operations were developed by Fukuda (1940) and Piepho (personal communication).

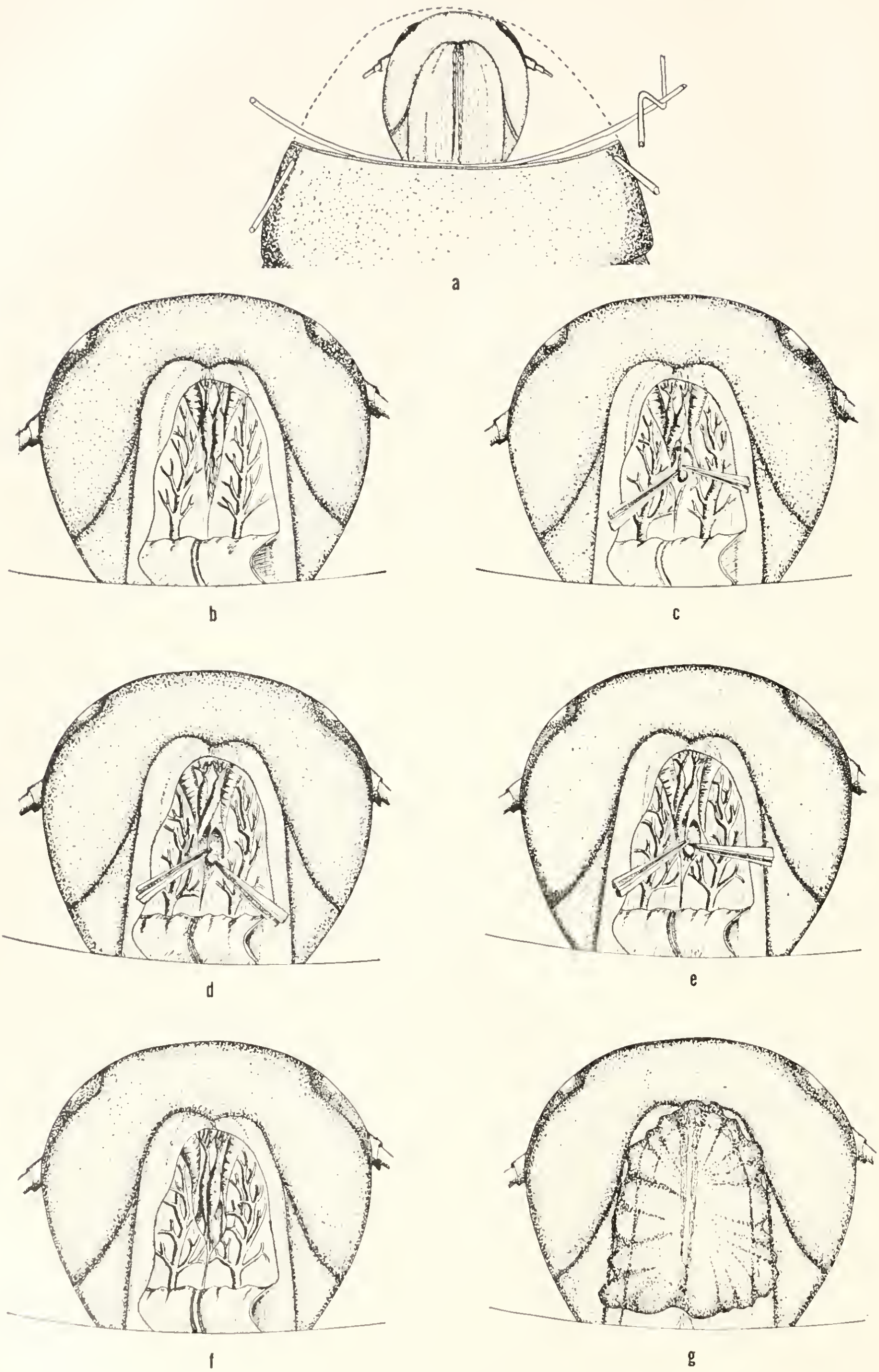


FIGURE 1. Technique for allatectomy of Blattaria. Description in text.

Fourth instar *Galleria* larvae, less than two days post-molt, were used in all experiments. Larvae were fully anesthetized with carbon dioxide for 20 to 30 minutes and the operation carried out under that gas. The outline of the operation for *Galleria* was that of Piepho.

A transverse dorsal incision was made immediately posterior and parallel to the pronotum; the pronotum was then pulled forward and pressed ventrally with forceps, exposing the esophagus. Tracing the esophagus anteriorly, the two very small, spherical corpora allata were located posterior to the brain. Each of these glands was removed individually by grasping the corpus cardiacum just anterior to the corpus allatum and by pinching the corpus cardiacum until the corpus allatum was free. The wound was closed with a layer of beeswax and petroleum jelly. During a 24-hour post-operative period the larvae were kept at 5° to 15° C. to reduce the number of deaths due to bacterial infections. At the end of 24 hours the larvae were apparently in better physiological condition to cope with normal bacterial contaminants and were returned to the culture rooms.

Bombyx mori was also anesthetized with carbon dioxide. Allatectomy was performed in a fashion similar to that used for *Galleria*. Third instar larvae, less than 24 hours post-molt, were used.

A transverse ligature of small polyethylene capillary tubing (0.61 mm. O.D.) was placed posterior to the third pair of thoracic legs to restrict the blood flow. A small incision was made anterior and above the first pair of thoracic legs on the latero-ventral surface of the thorax between the head capsule and the thorax. By retracting the edges of the incision, blotting the excess hemolymph, and freeing the area of some small tracheae, the homolateral corpus allatum was exposed lying ventral and posterior to the brain. An identical incision was made on the opposite ventro-lateral surface and the corpus allatum removed from that side. The wounds were sealed with the beeswax-petroleum jelly mixture and the operated animals placed in a polystyrene refrigerator box with a fresh supply of mulberry leaves.

Implants of Nosema

Implants were prepared from newly molted fifth instar *Tribolium* larvae which had been surface-sterilized and ligated anterior to the rectum and posterior to the brain. The terminal portions were discarded and the gut removed through a longitudinal incision in the body wall. The remainder of the larva was used as the implant. On some occasions *Nosema*-bearing fat body from *Tribolium* was introduced with a 26-gauge needle (Fisher and Sanborn, 1962).

Implantation procedures

Methods for introducing *Nosema* implants into cockroaches and lepidopteran larvae have been described previously (Fisher and Sanborn, 1962). To avoid introduction of the parasite into the hemocoel of cockroach hosts, the parasites were confined to glass chambers affixed to the pronotum. Two such chambers were used. The first was a capillary tube fixed into a small hole excised in the pronotum. "Kimax" capillary tubing (1.2 to 1.5 mm. O.D.) was cut into 2- to 5-mm. lengths and the ends polished on a carborundum stone to remove spurs. The finished tubes were then immersed in a zephiran solution for sterilization. A small piece of the exoskeleton was removed on the left side of the pronotum,

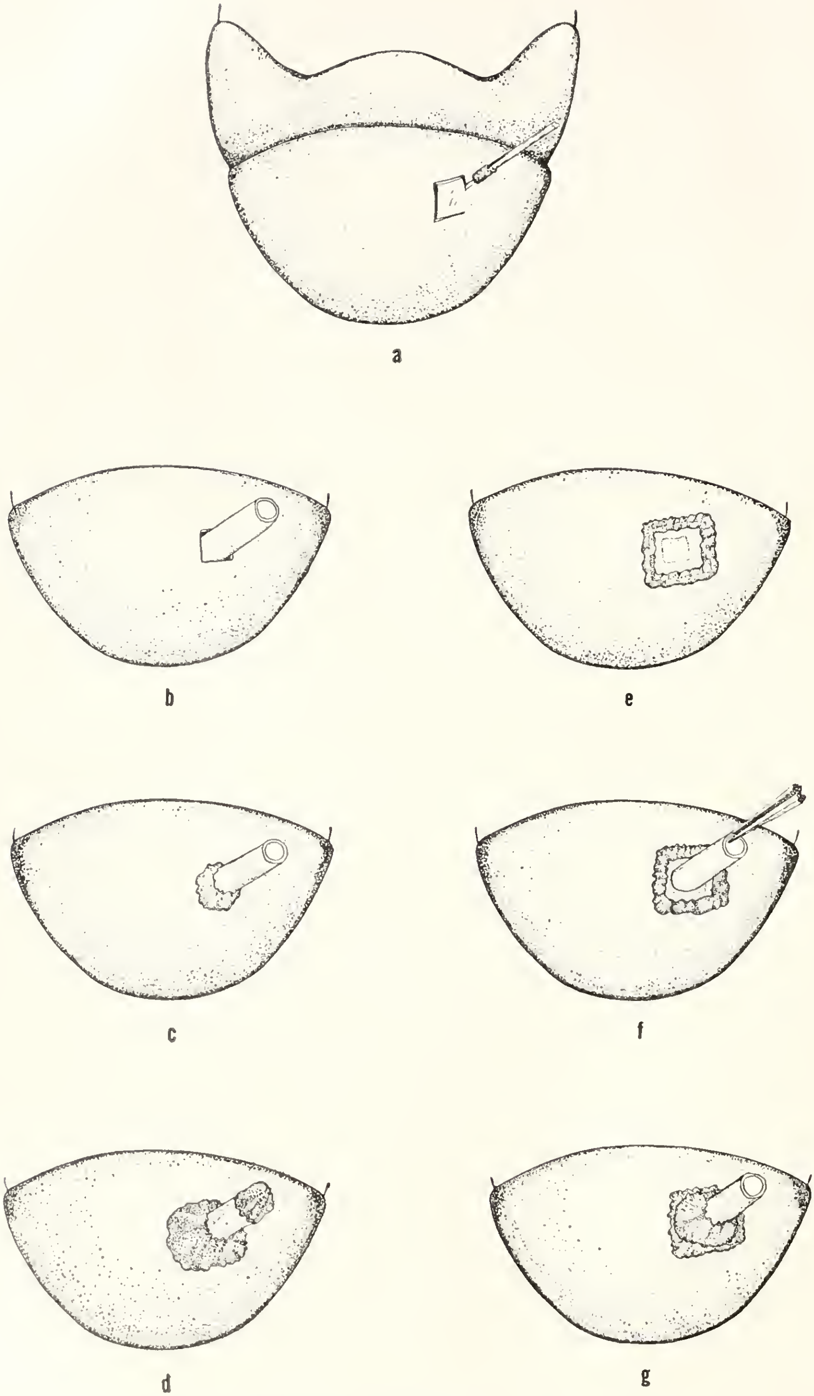


FIGURE 2. Techniques for attaching pronotal chambers to Blattaria. Description in text.

exposing the underlying tissue (Fig. 2, a). The tube was placed into the aperture and sealed in place with the petroleum jelly-beeswax mixture (Fig. 2, b and c). Gentle pressure was applied to the thoracic region of the roach, whereupon the blood rose in the tube. The implants were then placed in the tube in contact with the hemolymph, and the top of the tube sealed with wax (Fig. 2, d). Care was taken to exclude all air bubbles, which caused the death of an animal if present. To isolate the parasite from the host and to prevent infiltration of the host tissue, a second chamber method was employed. A 3- to 4-mm. square of Millipore (Millipore Filter Corp., Bedford, Mass.) filter (Type HA, pore diameter 0.45 μ) was placed over the excised hole in the pronotum and the edges sealed with petroleum jelly and beeswax (Fig. 2, e). Care was exerted to exclude all air bubbles before sealing the filter in position. The tube was then placed on the filter over the hole in the pronotum and sealed in place with the wax mixture (Fig. 2, f and g).

This tube arrangement cannot be filled with hemolymph by the technique used with unfiltered chambers, so they were filled by collecting blood from a severed antenna in a micro pipette and placing it into the attached chamber. The implant was added as before and the tube sealed with wax.

Preparation of Nosema spore extracts

Forty-eight g. of dried *Tribolium* larvae heavily infected with *Nosema* were homogenized with ether-washed sand in a blender. This dry homogenate was passed through a graded series of screens, the smallest mesh measuring 0.105 mm.

Eight grams of material which passed through the smallest screen contained spores of the parasite, particles of sand and host tissue. To remove the foreign material, the above material was suspended in 50 ml. of 1.0 *M* sucrose. Following centrifugation at 500 *g* for four minutes to remove particles of sand, the supernatant was recentrifuged at 1000 *g* for ten minutes to remove spores. Smaller particles of the host tissue remained in the supernatant. The primary pellet was rapidly washed by centrifugation three times in de-ionized water to remove the sucrose and dried *in vacuo* at 40° C. The resulting three grams were ground in a mortar with ether-washed Pyrex glass for about 15 minutes, to rupture the spore cases. This preparation was extracted with 400 ml. of diethyl ether and the spore-glass mixture decanted into a Buchner funnel and washed with an additional 200 ml. of diethyl ether. The supernatant and filtrate were combined and filtered through Whatman #1 filter paper. An additional 25 ml. of diethyl ether were used to wash the filter. This filtrate was evaporated to a volume of 200 ml. and washed with several volumes of saturated sodium chloride solution (Gilbert and Schneiderman, 1960). The final washed layer was evaporated to the residual oil which was dried *in vacuo* at 40° C. for several hours. Approximately 11 mg. of a viscous yellow oil were obtained from this preparation.

The complete procedure was repeated employing 32 g. of uninfected *Tribolium*. This preparation produced about 24 μg. of residue and served as the control for the extract from *Nosema*. The extracted materials were examined by gas chromatography according to the techniques described by Bates *et al.* (1961).

RESULTS AND DISCUSSION

1. *Nosema per os to Tribolium*

Normal uninfected *Tribolium* larvae show a progressive increase in weight until the nineteenth day, after which growth ceases. Pupation follows sometime between the twentieth and twenty-fifth day under our laboratory conditions. We confirm West's (1960) observations that *Tribolium* larvae infected with *Nosema* are larger in size and seldom pupate. If *T. castaneum* larvae are infected with *Nosema* on the fourth larval day, then removed from the infected flour after 24 hours' exposure and placed on clean flour, they increase in weight until the twelfth day. After this time there is a more rapid rate of increase in body weight until the twenty-fourth day, whereupon the weight diminishes until death which usually occurs after 30 days of larval life (Fig. 3). Growth curves presented in that figure are based on the averages of 80 animals in four separate experiments.

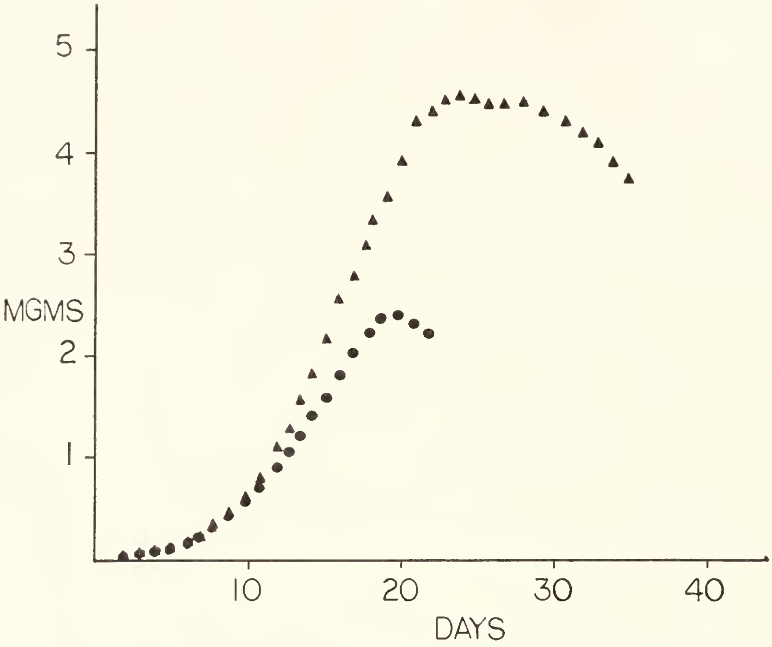


FIGURE 3. Growth curves of *T. castaneum* larvae: controls (●) termination in pupation, infected (▲) terminating in death.

Since the infected larvae attain twice the weight of the controls, we wished to see if there were any differences in the number of larval instars between these two groups. Individual *Tribolium castaneum* eggs were placed in creamers with approximately two grams of whole-wheat flour medium. After infection with *Nosema* on the fourth larval day, counts of the number of exuviae were made every five days until pupation, death of the larvae, or 40 days. On the basis of 20 such larvae in each of four groups we found that the control groups averaged seven to eight larval molts, while the infected larvae averaged 12 larval molts. None of the infected larvae pupated.

The observations on *Tribolium* lead us to believe that *Nosema* stimulated growth in these hosts. The larger size of the larvae and the occurrence of supernumerary molts indicated prolongation of the larval stage; this, it appeared to us, was affirmative evidence for the involvement of the parasite in the mechanism that controls the host's juvenile development.

Our observations were similar to those of Radtke (1942) who produced similar giant larvae in the closely related genus, *Tenebrio*, by transplanting the brain and the attached corpora allata from first instar larvae to early last instar larvae. Under the influence of hormones from the implanted tissues, the recipient larvae underwent as many as four supernumerary molts.

It would be convenient if all the experiments necessary to define the physiological relation of this parasite to the host could be carried out with the normal host. The anatomical position of the corpora allata made it impossible to allatectomize these insects. In *Tribolium* the corpora allata are located with the post-cerebral ganglion which cannot be surgically separated; hence, the larvae always died following such an operation. In view of this difficulty, we decided to see if the nosematid from *Tribolium* was infective for other insects in which allatectomy was possible. The results of the infection of hosts other than *Tribolium* have been discussed with regard to specificity in another paper (Fisher and Sanborn, 1962). In brief, it was found that *per os*, *Nosema* was not consistently infective to hosts anatomically amenable to allatectomy; however, a wide range of hosts could be infected surgically by the use of *Nosema* implants. From these observations crucial experiments were designed to see if the parasite had any effect on the host's development.

2. *Operations and Nosema implants—cockroaches*

Convenient species in which allatectomy is possible and in which we have previously shown that *Nosema* multiplies are provided by the Blattaria (Fisher and Sanborn, *loc. cit.*). Under our cultural conditions newly molted nymphs of *Blaberus craniifer*, weighing between 0.45 and 1.0 g. (fifth or sixth stage nymphs), normally molted to the next nymphal stage in about 32 days. With various sham operations the intermolt period was extended to 36 days. These operations included sham allatectomy, operations where one corpus allatum was removed, and sham allatectomy with the various procedures for implantation. The development period resulting from these sham operations overlapped considerably and did not differ significantly from the non-operated controls. Similar allatectomized nymphs molted in 56 days to stages which possessed adult-like characters. Scharrer (1946) has called these experimentally produced stages adultoids. When corpora allata were transplanted to various sites in the cockroach body, the cockroaches molted to normal nymphs in about forty days. Table I shows the effect of different operations on nymphs of this species.

The next series of experiments was concerned with allatectomized insects in which implants of *Nosema* from *Tribolium* were placed in the body cavity of the prospective host. In all such operations the prospective host was first allatectomized; then the implant was prepared and introduced.

TABLE I

Effect of operations on development of Blaberus craniifer

Treatment	Number of animals	Days to molt		State of roach after molt
		Range	Mean	
None	24	28–42	31.8	Nymphal
Sham operations	42	27–46	36.2	Nymphal
Allatectomy and corpora allata transplant	21	35–53	39.7	Nymphal
Allatectomy	52	44–68	55.5	Adultoid

Unoperated nymphs of *Blaberus* molted approximately every 32 days, whereas sham-operated control animals required 39 days. These controls were sham-allatectomized; the cockroach abdomen was opened and the fat body probed with forceps prior to closure of the wound. Allatectomized cockroaches, those which were allatectomized with the above abdominal operation, and those allatectomized and implanted with a *Nosema*-less implant, molted to adultoids in 56 days. If allatectomized animals were recipients of infected fat body of *Tribolium* or of transplanted corpora allata, another nymphal stage was produced at the next molt (Table II).

Developmental periods for sham-allatectomized nymphs, those allatectomized with the corpora allata transplanted to the abdomen of the cockroach, and allatectomized nymphs with implants were all close to 40 days. Nymphs which were allatectomized, allatectomized in addition to an abdominal sham operation, and those with a *Nosema*-less implant all developed to adultoids in approximately 56 days. These observations indicate that *Nosema* replaces the corpora allata of *Blaberus*, inasmuch as the cockroach does not become adultoid upon allatectomy, and that the intermolt period is the same as in allatectomized cockroaches with corpora allata transplanted to their abdomen.

Table III shows that *Byrsotria fumigata* behaved similarly. Only a few animals of this species were available for experiments. Control animals molted in about 34 days; sham-operated controls required 56 days. Adultoids were produced from allatectomized nymphs in about 63 days while allatectomized nymphs with implants of *Nosema* molted to another nymphal stage in about 55 days.

TABLE II

The effect of Nosema *on* Blaberus craniifer. *Abdominal implants*

Treatment	Number animals	Days to molt		State of roach after molt
		Range	Mean	
None	24	28–42	31.8	Nymphal
Sham operations	6	27–39	39.0	Nymphal
Allatectomy and implants of *Nosema* or corpora allata	19	32–59	39.7	Nymphal
Allatectomy or allatectomy and sham or *Nosema*-less implants	62	44–68	56.0	Adultoid

TABLE III

The effect of Nosema *on* Byrsotria fumigata. *Abdominal implants*

Treatment	Number of animals	Days to molt		State of roach after molt
		Range	Mean	
None	3	27–41	33.6	Nymphal
Sham operations	4	52–60	56.5	Nymphal
Allatectomy and implant of *Nosema*	5	52–57	55.0	Nymphal
Allatectomy	4	55–75	63.5	Adultoid

The intermolt period of this species is greatly prolonged following sham operations. Again the results of the operations were clear-cut. The ablation of the corpora allata produced an adultoid, while *Nosema* implants prevented adult development and a nymphal stage resulted.

These cockroaches require a much longer intermolt period following operation. The cuticle of *Byrsotria* is much heavier than that of the other species, and due to the morphology of the pronotum a larger portion of that structure must be removed to conduct the allatectomy. Since the molting of insects is delayed when injury is present, this is in all probability the cause of the prolonged intermolt period following operations with this species. Despite the exaggerated intermolt period following any operation, allatectomized nymphs were adultoid at the next molt.

Nymphs weighing between 0.30 and 0.35 g. normally molted in approximately 29 days. When this size nymph (fifth or sixth instar) was sham-allatectomized, the individuals molted, yielding the next nymph, in about 34 days. When they were allatectomized, an adultoid was produced at the next molt, which occurred in approximately 43 days. When *Tribolium* fat body without *Nosema* was introduced, the host molted to an adultoid stage in 40 days, whereas those with such implants with *Nosema* molted to nymphs in about 39 days. There was a high mortality following operations on this species. These experiments are summarized in Table IV.

TABLE IV

The effect of Nosema *on* Periplaneta americana. *Abdominal implants*

Treatment	Number of animals	Days to molt		State of roach after molt
		Range	Mean	
None	6	26–35	28.9	Nymphal
Sham allatectomy, one-half allatectomy	8	31–37	34.0	Nymphal
Allatectomy and *Nosema* implant	3	38–41	39.3	Nymphal
Allatectomy and *Nosema*-less implant	5	37–42	40.0	Adultoid
Allatectomy, allatectomy and sham operation	18	32–56	42.9	Adultoid

3. Operations and Nosema implants—Lepidoptera

Fourth instar *Galleria* larvae, allatectomized within 24 hours following a molt, metamorphosed prematurely to pupae in about 17 days. Controls molted to the fifth instar larvae in approximately 12 days under our laboratory conditions.

Allatectomized 12-hour-old third instar larvae of *Bombyx mori* metamorphosed to small pupae in about ten days; comparable control larvae normally molted to fourth instar larvae in six to eight days.

The effects of *Nosema* implants on the development of *Bombyx mori* are worth noting. When third instar larvae were allatectomized within 12 hours following the second molt, miniature pupae were produced at the next molt. Allatectomized fourth instar larvae whose corpora allata were supplemented with a *Nosema*-containing implant developed into what appeared to be normal pupae. On one occasion an allatectomized larva with a *Nosema* implant underwent a supernumerary molt and produced a giant pupa. Due to limited numbers of such larvae we could not conduct further studies on this species; nevertheless, these observations afforded evidence that *Nosema* had a physiological action in Lepidoptera similar to that which it has in Coleoptera and Blattaria.

4. External implantations

Evidence presented to this point indicated that *Nosema* was acting in some way to inhibit adult metamorphosis in the species of insects investigated. Since this parasite was placed directly in the fat body of the new host, it was conceivable that it acted by causing tissue damage to the host. To avoid such a possibility we chose to isolate the parasites in a chamber outside the host. This technique minimized tissue damage, but a passage remained open to the host through which materials from the parasite could pass into the host.

TABLE V

The effect of Nosema *on* Blaberus craniifer. *Pronotal chamber*

Treatment	Number of animals	Days to molt		State of roach after molt
		Range	Mean	
None	24	28–42	31.8	Nymphal
Sham operations	5	39–41	39.8	Nymphal
Allatectomy and corpora allata to pronotal chamber	5	35–50	42.5	Nymphal
Allatectomy and implant of *Nosema* to pronotal chamber	7	40–60	47.7	Nymphal
Allatectomy, allatectomy and sham operations, allatectomy and *Nosema*-less implant to pronotal chamber	60	44–70	55.9	Adultoid

Nymphs of *Blaberus craniifer* were used in these experiments. Table V shows the results obtained with chambers in which there was a direct opening to the host's hemocoel.

Normal controls for this group molted to the next nymphal stage in about 32 days. Sham-operated controls, and those nymphs with the corpora allata transplanted to the pronotal chamber, developed to the next stage nymph in about 40 days. Those nymphs which were allatectomized, allatectomized with a sham pronotal chamber, and allatectomized with a pronotal chamber containing non-infected *Tribolium* fat body developed into adultoids in approximately 56 days. If fat body containing *Nosema* was placed in such a chamber on an allatectomized host, a nymph was produced at the next molt in about 48 days. Microscopic examination of the tissues underlying the chambers showed limited infiltration of the parasite. Proliferation of the parasite was not as extensive, however, as in the abdominal implants.

Since there was some parasitic infiltration of the host tissue, the next step was to exclude the parasites from the host's body. Table VI shows the results of experiments where the chamber was separated from the hemocoel by a Millipore filter. The allatectomized nymphs with a chamber containing only blood, and those which received an implant of *Tribolium* fat body without *Nosema*, molted to adultoids in approximately 56 days. If *Nosema* was present in the *Tribolium* fat body, the next molt was nymphal and occurred in about 47 days.

TABLE VI

The effect of Nosema *on* Blaberus craniifer. *Pronotal chamber separated by filter*

Treatment	Number of animals	Days to molt		State of roach after molt
		Range	Mean	
None	24	28–42	31.8	Nymphal
Sham operations	4	37–40	39.0	Nymphal
Allatectomy and *Nosema* implant to chamber	5	40–56	47.1	Nymphal
Allatectomy, allatectomy and sham operation, allatectomy and *Nosema*-less implants	60	43–68	55.9	Adultoid

These observations confirm the fact that *Nosema* elaborates some substance with juvenile hormone activity. We could find no parasitic infiltration in the tissue below the filter, and the spores taken from the chamber at the conclusion of this experiment remained infective to *Tribolium*.

5. *Assay of Nosema spore extracts*

Taking advantage of the extreme sensitivity of regenerating epithelial tissue (Piepho, 1950; Piepho and Heims, 1952), the extracts were applied to areas of exposed epidermis on previously chilled *Hyalophora cecropia* pupae which had developed at room temperature for two days (Williams, 1956). Following the application of the extract, the exposed area was covered with a layer of melted paraffin wax. The extract of *Nosema* possessed juvenile hormone activity since there was retention of the pupal cuticle on the newly emerged adult moths where the extract was applied.

To further assay the activity of the *Nosema* extracts, the extract was placed in a chamber attached to the pronotum of an allatectomized *Blaberus* nymph. Corpora allata were transferred to chambers on similar hosts. The results are summarized in Table VII. Allatectomized nymphs with pronotal chambers containing only hemolymph molted to adultoids in 56 days as do allatectomized animals with chambers containing the control extract. When two corpora allata were placed in such a chamber, the cockroach molted to a nymph in approximately 43 days. When the extract of *Nosema* was added, the allatectomized nymph underwent a nymphal molt after 38 days.

TABLE VII

The effect of implanted corpora allata and extracts of Nosema on Blaberus craniifer. *Pronotal chamber*

Treatment	Number of animals	Days to molt		State of roach after molt
		Range	Mean	
None	24	28–42	31.8	Nymphal
Allatectomy and *Nosema* extract	3	34–41	37.7	Nymphal
Sham operations	5	39–41	39.8	Nymphal
Allatectomy and corpora allata transplant to pronotal chamber	6	35–53	42.5	Nymphal
Allatectomy, allatectomy and sham operation, allatectomy and control extract and pronotal tube	58	44–70	56.0	Adultoid

No really quantitative assay exists for the juvenile hormone. Williams (1959) injected ether extracts from the abdomens of male *Hyalophora cecropia* into chilled *Antheraea polyphemus* pupae. These pupae molted to form pupae which possessed both pupal and adult characteristics. He also applied those extracts to the exposed epidermis of chilled *H. cecropia* pupae and noted there was retention of pupal integument at the point of contact with the hormone. Extracts applied after the fourth day of adult development had no effect.

Wigglesworth (1958) proposed several methods for the assay of juvenile hormone, which were based on the extreme sensitivity of regenerating epithelial tissue to juvenile hormone. Gilbert and Schneiderman (1960) developed an assay for juvenile hormone, in which they scored the degree of metathetely achieved upon injection of the hormone into developing pupae of *A. polyphemus*.

Assays have not been of major interest in this investigation other than to show that *Nosema* extracts possessed juvenile hormone activity. The retention of juvenile integument by adult *H. cecropia* following topical application of the *Nosema* extract to pupae is a positive test for juvenile hormone according to the criteria of the above-mentioned assays. Further, this extract is capable of sustaining the nymphal state of *Blaberus* in the absence of its own corpora allata.

Thus, *Nosema in vivo* has a juvenilizing effect in that it can prevent adult development in the absence of the corpora allata of the host. The material responsible for the juvenilizing effect is extractable from the parasite spores and mimics juvenile hormone according to the criteria of generally accepted assays.

Examination of the extracted materials by gas chromatography did not disclose the presence of any farnesol-isomers, which have been shown to have juvenile hormone activity (Wigglesworth, 1961; Schmialek, 1961; Karlson, 1963).

Conclusions

In *Tribolium, Nosema* usually infects the fat body and occasionally portions of the nervous system (West, 1960). Two non-specific events occur: histolysis of the fat body tissue and hyperplasia of all cells in the area surrounding the parasites. More interesting is a specific effect on growth and differentiation of the insect, in which the parasite stimulates growth, but exerts a concomitant inhibition over differentiation to the adult state. The result is a larva of larger than normal size.

Giant immature stages of many insects have been produced by transplanting the brain and attached corpora allata or corpora allata of younger stages in the life history to the last juvenile stage. Wigglesworth (1936) with *Rhodinus*, Pflugfelder (1939) with *Dixippus*, and Pfeiffer (1945) with *Melanopus* elucidated this phenomenon in the hemimetabolous insects. Radtke (1942) and Piepho (1942) demonstrated the same phenomenon with the holometabolous species, *Tenebrio* and *Galleria*. Secretion of the corpora allata following such transplantation is responsible for extended juvenile development leading to supernumerary molts and larger than normal juvenile stages.

A similar phenomenon is observed when *Tribolium* larvae are infected with *Nosema*. They also undergo supernumerary molts, producing giant larvae which weigh twice as much as non-infected controls. These infected larvae do not develop past the larval state. Infection then mimics the effect of active corpora allata in prolonging larval life and preventing maturation.

To demonstrate that *Nosema* acts directly, and not by an indirect effect on the corpora allata of the host, the insect's corpora allata were removed and replaced with implants of *Nosema*. Under these circumstances a nymph is produced at the next molt, whereas allatectomized insects without *Nosema* produced adultoids. When the parasites were enclosed in a chamber open to the insect hemocoel, the same effect was obtained. If this chamber was separated from the insect with a Millipore filter, which excludes the parasite from the hemocoel, the identical response was observed.

To us, these observations clearly demonstrate the ability of the parasite to produce a substance with juvenile hormone activity. To confirm this hypothesis, we have extracted a substance from the parasite by techniques used for the purification of juvenile hormones from other tissues. This extract has the same biological properties in standard assays for juvenile hormone as do the extracts from insects.

It is not surprising that a substance possessing juvenile hormone activity can be obtained from the parasite, since Schneiderman and Gilbert (1957, 1958) and Williams *et al.* (1959) have found ether-soluble materials from sources as diverse as invertebrates representing 13 classes of most of the major phyla and from thymus, human placenta, and other mammalian organs. Schneiderman and Gilbert (1963) list 13 chemicals possessing some degree of juvenile hormone activity. Wigglesworth (*loc. cit.*), Schmialek (*loc. cit.*), and Karlson (*loc. cit.*) have all emphasized the importance of farnesol and related compounds as substances which occur in insects and have juvenile hormone activity in some test systems. While we have not tested all of the substances which have been shown to be active, we have found that extracts of *Nosema* do not contain any of the isomers of farnesol in an amount sufficient to explain their activity.

Although it is not unique to find active extracts, it is remarkable that a parasite is capable of influencing the normal growth and development of its host by producing a substance with activity identical to that of a hormone of the host.

Animal parasites are commonly associated with anemias, nutritional and metabolic disturbances and occasionally with endocrine malfunction of their hosts; to our knowledge, however, this is the first experimental approach to a problem concerning a parasite producing a substance with identical activity to an endogenous hormone of its animal host, and establishes a new parameter of the concepts of such host-parasite associations.

SUMMARY

1. In *Tribolium* the infection of *Nosema* stimulates growth of the host, but deters development to the adult state. Infected *Tribolium* larvae pass through as many as six supernumerary molts, resulting in giant larvae which weigh twice as much as uninfected controls.

2. Surgical transplants of *Nosema* to allatectomized Blattaria prevent adult development and a nymph is produced at the next molt. If the parasites are confined to a small chamber affixed to the nymph, but open to the nymph's hemocoel, the same effect is obtained. If such a chamber is separated from the host with a Millipore filter preventing migration of the parasites while allowing passage of the active principle, the allatectomized nymph does not metamorphose. These experiments prove that *Nosema* is capable of replacing the hormone normally produced by the host's corpora allata.

3. Parasite spores have been extracted by the techniques used to purify natural juvenile hormone and the resultant extract has juvenile hormone activity in tests performed on *Hyalophora cecropia* pupae and allatectomized *Blaberus craniifer*. The extracted material, however, does not have sufficient farnesol isomers to explain the juvenilizing effect.

LITERATURE CITED

ALTSON, A. M., 1920. The life history and habits of two parasites of blowflies. *Proc. Zool. Soc. London*, **1920**: 195–243.

BATES, B. B., D. M. GALE, B. V. GRUNER AND P. P. NICHOLAS, 1961. The stereoisomeric farnesols. *Chem. Ind.* (London) 1907–1908.

BODENSTEIN, D., 1953. Studies on the humoral mechanism in growth and metamorphosis of the cockroach, *Periplaneta americana*. II. Function of the prothoracic gland and corpus cardiacum. *J. Exp. Zool.*, **123**: 413–431.

BODENSTEIN, D., 1954. Endocrine mechanisms in the life of insects. *Recent Prog. Hormone Research*, **10**: 157–182.

FINLAYSON, L. H., AND V. ANN WALTERS, 1957. Abnormal metamorphosis in saturnid moths infected by a microsporidian. *Nature*, **180**: 713–714.

FISHER, F. M., JR., AND R. C. SANBORN, 1962. Observations on the susceptibility of some insects to *Nosema* (Microsporidia, Sporozoa). *J. Parasitol.*, **48**: 926–932.

Fukuda, S., 1940. Hormonal control of molting and pupation in the silkworm. *Proc. Imp. Acad. Tokyo*, **16**: 417–420.

Gilbert, L. I., and H. A. Schneiderman, 1960. The development of a bioassay for the juvenile hormone of insects. *Trans. Amer. Micr. Soc.*, **79**: 38–67.

Halcrow, J. G., 1954. The phenomenon of metathetely, with notes on the parasites of some Mauritian mosquitoes. *Entomol.*, **87**: 122–123.

Karlson, P., 1963. Chemistry and Biochemistry of Insect Hormones. *Angewandte Chem.* (Intl. Ed. in English), **2**: 175–182.

Keilin, D., and V. C. Robinson, 1933. The morphology and life history of *Aproctonema entomophagum* Keilin, a nematode parasite in the larvae of *Sciara pullula* Winn. (Diptera-Nematocera). *Parasitol.*, **25**: 285–295.

Pfeiffer, I. W., 1945. The influence of the corpora allata over development of nymphal characters in the grasshopper *Melanoplus differentialis*. *Trans. Conn. Acad. Arts Sci.*, **36**: 489–513.

Pflugfelder, O., 1939. Beeinflüssung von Regenerationsvorgängen bei *Dixippus morosus* Br. durch Extirpation und Transplantation der Corpora allata. *Zeitschr. wiss. Zool.*, **152**: 159–184.

Piepho, H., 1942. Untersuchungen zur Entwicklungs-Physiologie der Insekten-metamorphose—Ueber die Puppenhäutung der Wachsmotte *Galleria mellonella* L. *Arch. f. Entw.*, **141**: 500–583.

Piepho, H., 1950. Über die Hemmung der Falterhautung durch Corpora allata. Untersuchungen an der Wachsmotte *Galleria mellonella* L. *Biol. Zentralbl.*, **69**: 261–271.

Piepho, H., and A. Heims, 1952. Das Kutikulamuster der Schmetterlingslarve und die hormonal Grundlage seiner Entstehung-Untersuchungen an der Wachsmotte *Galleria mellonella* L. *Zeitschr. f. Naturforsch.*, **76**: 231–237.

Radtke, A., 1942. Hemmung der Verpuppung deim Mehlkäfer, *Tenebrio molitor* L. *Naturwiss.*, **29**: 451–452.

Salt, G. 1932. The natural control of the sheep blow fly, *Lucilia sericata* Meigen. *Bull. Ent. Research*, **23**: 235–245.

Scharrer, B., 1946. The relationship between corpora allata and reproductive organs in adult *Leucophaea maderae* (Orthoptera). *Endocrinol.*, **38**: 46–55.

Scharrer, B., 1955. Hormones in Invertebrates. *In:* G. Pincus and K. V. Thimann, Eds., *The Hormones*, **3**: 57–95. Academic Press, Inc., New York.

Schmialek, P., 1961. Die Identifizierung zweier im Tenebriokot und in Hefe vorkommender Substanzen mit Juvenilhormonwirkung. *Zeitschr. f. Naturforsch.*, **16B**: 461–463.

Schneider, F., 1950. Die Entwicklung Syrphid parasiten *Diplazon fissorius* Grav. (Hymen., Ichnem.) in uni, oligo- und polyvoltinen Wirten und sein Verhalten bei parasitärer Aktivierung der Diapauselarven durch *Diplazon pectoratorius* Grav. *Mitteh. Schweiz. Ent. Gesellsch.*, **23**: 155–194.

Schneider, F., 1951. Einige physiologische Beziehungen zwischen Syrphidenlarven und ihren Parasiten. *Zeitschr. angew. Ent.*, **33**: 150–162.

Schneiderman, H. A., and L. I. Gilbert, 1957. The distribution of chemical properties of the juvenile hormone of insects. *Anat. Record*, **128**: 618.

Schneiderman, H. A., and L. I. Gilbert, 1958. Substances with juvenile hormone activity in Crustacea and other invertebrates. *Biol. Bull.*, **115**: 530–535.

Schneiderman, H. A., and L. I. Gilbert, 1963. Control of growth and development in insects. *Science*, in press.

Strickland, E. H., 1911. Some parasites of *Simulium* larvae and their effects on the development of the host. *Biol. Bull.*, **21**: 302–338.

Van der Kloot, W. G., 1960. Neurosecretion in insects. *Ann. Rev. Ent.*, **5**: 35–52.

Van der Kloot, W. G., 1962. Comparative endocrinology of invertebrates. *Ann. Rev. Physiol.*, **24**: 491–516.

Varley, G. C., and C. G. Butler, 1933. The acceleration of development of insects by parasitism. *Parasitol.*, **25**: 263–268.

West, A. F., 1960. The biology of a species of *Nosema* (Sporozoa: Microsporidia) parasitic in the flour beetle *Tribolium confusum*. *J. Parasitol.*, **46**: 747–754.

Wigglesworth, V. B., 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci.*, **79**: 91–121.

WIGGLESWORTH, V. B., 1958. Some methods for assaying extracts of juvenile hormone in insects. *J. Ins. Physiol.*, **2**: 73–84.

WIGGLESWORTH, V. B., 1961. Some observations on the juvenile hormone effect of farnesol in *Rhodnius prolixus* Stål (Hemiptera). *J. Ins. Physiol.*, **7**: 73–78.

WILLIAMS, C. M., 1956. The juvenile hormone of insects. *Nature*, **178**: 212–213.

WILLIAMS, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult cecropia silkworm. *Biol. Bull.*, **116**: 323–338.

WILLIAMS, C. M., L. V. MOORHEAD AND J. F. PULIS, 1959. Juvenile hormone in thymus, human placenta and other mammalian organs. *Nature*, **183**: 405.

AN AIR-ABRASIVE METHOD FOR THE MICRODISSECTION OF MINERALIZED TISSUES[1]

ULF A. FRIBERG,[2] PHILIP T. LEVINE AND MELVIN J. GLIMCHER[3]

Department of Orthopedic Surgery, Harvard Medical School and The Massachusetts General Hospital, and the Forsyth Dental Center, Boston 14, Massachusetts

Biologically mineralized tissues are often composed of several histologically distinct tissue layers containing inorganic crystals of different structure, size, shape, and orientation, and organic matrices of varying composition and structure. In order to study the molecular structure, organization, and composition of the inorganic crystals and the organic matrices of the individual layers by both biophysical and biochemical methods, it is necessary that the individual histological entities be separated without contamination from adjoining layers, which in many cases interdigitate at their junction. Furthermore, it may also be necessary, in the case of external or exposed surfaces, to remove extraneous deposits or layers of material covering the surface without the use of chemical agents, which may alter the underlying inorganic crystals, or leach out various soluble organic components. In order to obtain such histologically defined material, the samples must be prepared by microdissection. This is a difficult technical task, however, because of the extreme hardness and the firm adherence of the interdigitating layers.

The necessity of obtaining histologically characterized tissue and the problems involved in the preparation of such samples are well illustrated by reference to one of the current projects under investigation in these laboratories, the characterization of the structural proteins of mature, erupted, bovine dental enamel.

Since the enamel of mature bovine teeth contains approximately 0.06% protein by weight (Glimcher, Friberg and Levine, unpublished data) and is situated between a layer of dentin and cementum (Glimcher, Friberg and Levine, 1963), each of which contains approximately 30% protein (primarily collagen) by weight, a minute contamination from either source in the enamel sample would result in a very serious and misleading error in an analysis of the organic matrix of enamel. For example, 70% to 80% of the proteins derived from a sample of enamel containing 1% dentin or cementum would contain 70% to 80% collagen and only 20% or 30% enamel proteins. In order to characterize the enamel proteins, it is

---

[1] Supported by research grants from the U. S. Public Health Service (DE-01777 and AM-06375-02) and the John A. Hartford Foundation, Inc. to the Harvard Medical School and the Massachusetts General Hospital, and from the American Chicle Company and the Colgate-Palmolive Company to the Forsyth Dental Center.

[2] Awardee of an International Postdoctoral Research Fellowship (FF-267) National Institutes of Health. Present address: Department of Histology, Karolinska Institute, Stockholm 60, Sweden.

[3] Communications should be addressed to: Melvin J. Glimcher, M.D., Orthopedic Research Laboratories, Massachusetts General Hospital, Boston 14, Massachusetts.

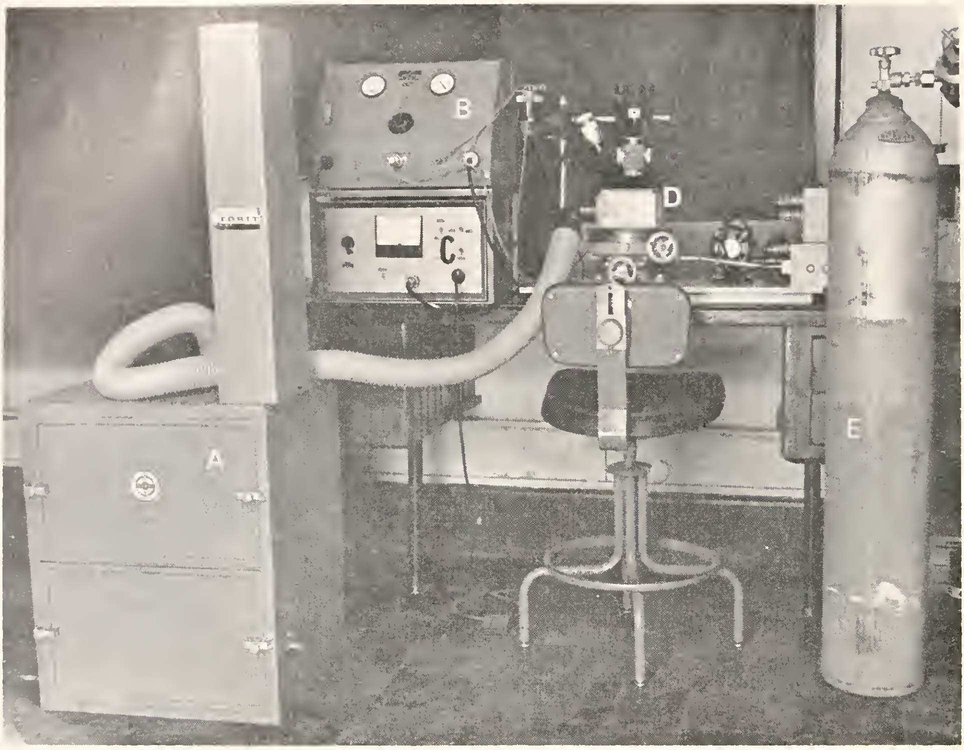


FIGURE 1. Overall view of apparatus: (A) dust collector; (B) air-abrasive unit over (C) master control unit; (D) microdissection unit; (E) gas cylinder.

therefore essential that the samples be free of the adjacent layers of dentin and cementum. Furthermore, because of the very low protein content of the enamel, large amounts of tissue are required for the analyses.

In this report, a method is described for the sectioning of enamel and other mineralized tissues, employing the principle of air-abrasive cutting. Sectioning of the fully mineralized samples is accomplished by feeding compressed air or gas through a vibrating chamber filled with abrasive powder and directing the resultant air-abrasive stream at supersonic speed through a fine nozzle at the object to be cut. This technique is widely used in industry to provide cool, non-contact cutting of materials such as mica, glass, crystals, ceramics, refractory metals, and others that would be likely to shatter with the usual contact methods. In a recent non-industrial application this procedure has been used to uncover rock-embedded fossil material (Stucker, 1961).

In order to employ the air-abrasive method of cutting for the microdissection of mineralized tissues a special apparatus was designed and constructed. This apparatus allows mineralized tissue sections to be mounted on a motorized cross-feed stage and passed under direct microscopic viewing below a fixed nozzle delivering the air-abrasive jet.

Experimental

Description of the apparatus

An overall view of the apparatus is presented in Figure 1. It consists of the following 5 subunits: air-abrasive unit, master control unit, microdissection unit, dust collector, and gas source.

The air-abrasive unit shown in Figure 2 (Model C Industrial Air-abrasive Unit, S. S. White) is used without internal modification. It is placed on a shelf over the master control unit (Fig. 2) to which all external electrical connections are made. The master control unit consists of various controls and two D. C. supply units; the air-abrasive unit and the dust collector are connected to the mains through it. One of the D. C. supply units delivers a fixed voltage of 120 V to relay coils and electromagnetic clutches as well as to the field winding of the table-drive shunt motor. The armature winding is connected to the second D. C. supply, which is adjusted from 0 to 120 V through the use of a variable auto-transformer. The output speed of the motor is proportional to the armature voltage, which is indicated by a volt-meter. Positioning of the specimen table at full armature voltage is effected through two, mutually exclusive push-buttons. Table direction during the cutting is preset with a toggle switch and the speed is set by means of the

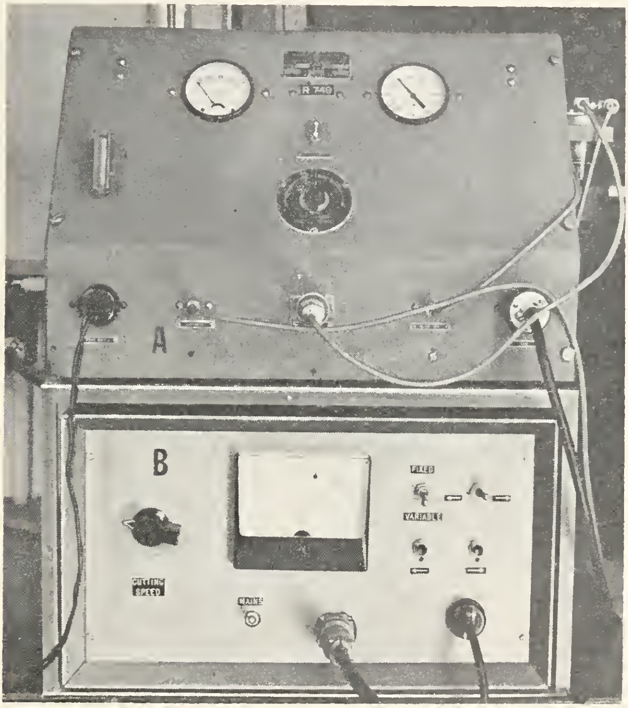


Figure 2. Air-abrasive unit (A) mounted above master control unit (B). Individual controls are labeled.

variable transformer. During microdissection, the motorized table-drive is controlled through a foot-switch, which simultaneously activates the air-abrasive stream and the dust collector.

The ~~*microdissection*~~ Sectn holder. *unit* consists of a specimen stage and a stereo microscope mounted on a steel base together with the drive motor and a two-speed gearbox (Fig. 3). A cross-feed rotary milling table, which is horizontally mounted, serves as the specimen stage. The section holder is placed on top of this unit. The section holder is made of a one-half inch brass block with a spring clamp in a dust enclosure box (Fig. 4). Horizontal motion of the table (from left to right, or right to left) is effected by means of the shunt motor. The crank wheels for the cross-feed and table rotation are controlled manually. One full turn of the crank-wheel corresponds to 0.1″ travel or 9° rotation, respectively.

The dust enclosure box (Figs. 3, 4) is made of acrylic resin except for a glass top, which can be replaced when vision becomes impaired due to the action of the

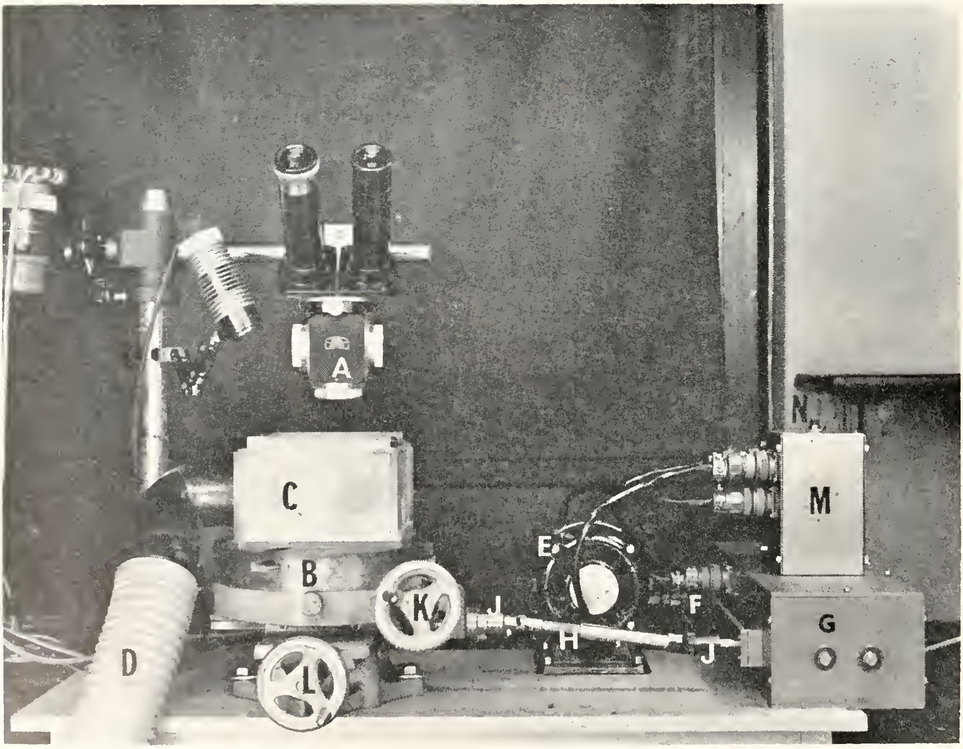


FIGURE 3. Microdissection unit: The stereo microscope (A) is mounted above the cross-feed stage (B). The dust enclosure box (C) contains the specimen holder and is connected to the collector hose (D). The drive motor (E) is connected through a flexible shaft coupling (F) to the input shaft of the two speed gearbox (G). The output of the gearbox is connected to the specimen table by an extension shaft (H) and universal joints (J). The wheel cranks (K) and (L) manually control rotary and fore-and-aft motion of the specimen table during dissection. The coupling box (M) is used to disconnect the motor temporarily when making right-angle end cuts by means of the push button (N).

abrasive. The floor of the dust enclosure is protected by a piece of hard rubber. The hand piece, which extends into the dust enclosure box through a rectangular opening in the rear wall, is mounted so that the nozzle tip is tilted a few degrees from a vertical position. This permits the operator to observe the end of the nozzle tip and its position in relation to the specimen through the stereo microscope during the actual dissection. A metal wire mesh over the vacuum suction opening prevents loss of dissected specimens. In order to prevent the air-abrasive stream from cutting the section holder, the tissue sections are mounted so that they extend ½ inch over the edge of the brass block. Illumination of the sections during microdissection is provided by an overhead lamp, which is mounted (as is the stereo microscope) on a vertical steel rod.

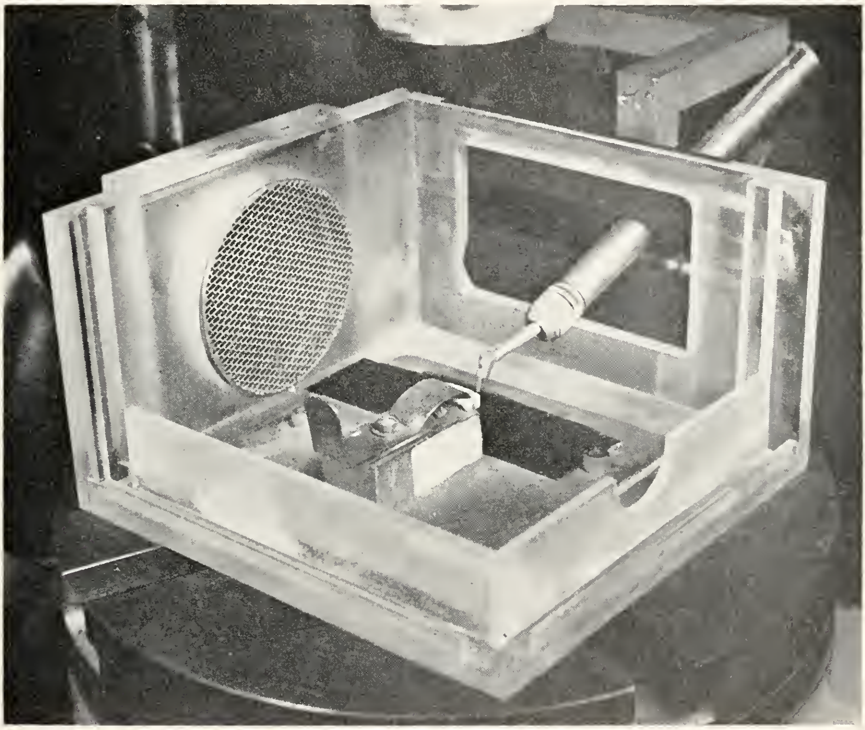


FIGURE 4. Cross-section of tooth crown mounted in dust enclosure box with nozzle positioned for cutting.

The handpiece (Fig. 4), with a right-angle nozzle tip, is mounted in a fixture and may be raised and lowered through a rack and pinion drive (Fig. 4). A variety of interchangeable nozzle tips are available with rectangular and circular orifices and with tip angles of 90°, 135°, and 180°. The rectangular orifice nozzles are available as 0.003″ × 0.060″ or 0.006″ × 0.060″. Circular orifice nozzles are made with 0.010″, 0.018″, and 0.026″ diameter. A rectangular orifice nozzle is used when narrow, rectilinear cuts are required. The minimum width of the cutting

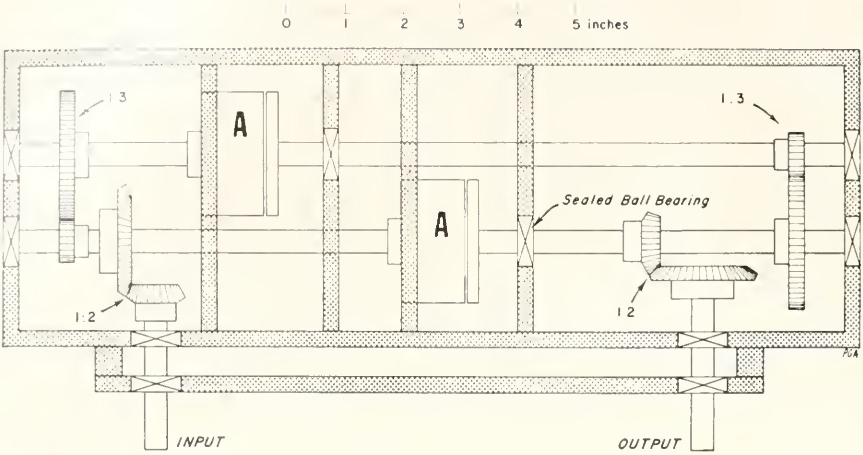


Figure 5. Two speed gearbox with electromagnetic clutches (A) for remote control.

path is approximately 100 to 200 microns for the rectangular orifice nozzles, and 300, 500, and 700 microns for the circular orifices. For curvilinear cutting, as in the preparation of enamel, the use of nozzles with circular orifices is preferable.

The drive motor is a 1/15 hp D. C. shunt motor with a 1:10 worm gear and a nominal output speed of 173 rpm (Bodine NSH-34RH). It is connected through a flexible shaft coupling to the input shaft of the two-speed gearbox. The latter is built from ¼″ sheet brass and has 24 pitch bronze gears and ⅜″ shafts running in sealed ball bearings (Fig. 5). The gear ratios of 1:4 and 1:36 are remotely selected through two electromagnetic clutches (Sterans 2.5 SMR). The 1:4 ratio

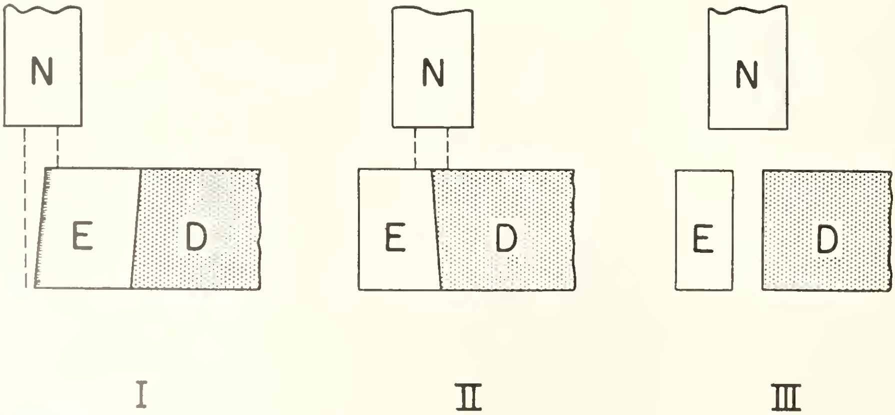


Figure 6. Diagramatic representation of the position of the specimen in relation to the nozzle (N) during dissection. I. Removal of cementum (horizontal shaded surface) and outer one-fourth of the enamel (E). II. Position of the nozzle at the junction of dentin (D) and enamel (E) after specimen has been turned over. Note that the inner one-fourth of the enamel is also sacrificed. III. Slab of enamel fully dissected.

is used with full motor speed for pushbutton positioning of the table. The 1:36 ratio is used for cutting in conjunction with a variable motor speed. Under prevailing load conditions, the gearbox output is 50 rpm at positioning, and 0.7 to 5.5 rpm at cutting, corresponding to table speeds of 5 ipm and 0.07 to 0.55 ipm, respectively. The output shaft of the gearbox is connected to the specimen table through universal joints and an extension shaft. Electrical connections from the master control unit to motor and gearbox run through a coupling box and are used to disconnect the motor temporarily when making right-angle end cuts.

To prevent the abrasive powder from being disseminated throughout the work area, the dust enclosure is connected through a flexible hose to an industrial dust collector (Torit Model 54). Noise and vibration have been minimized by fitting the dust collector with an exhaust silencer and shockmounts.

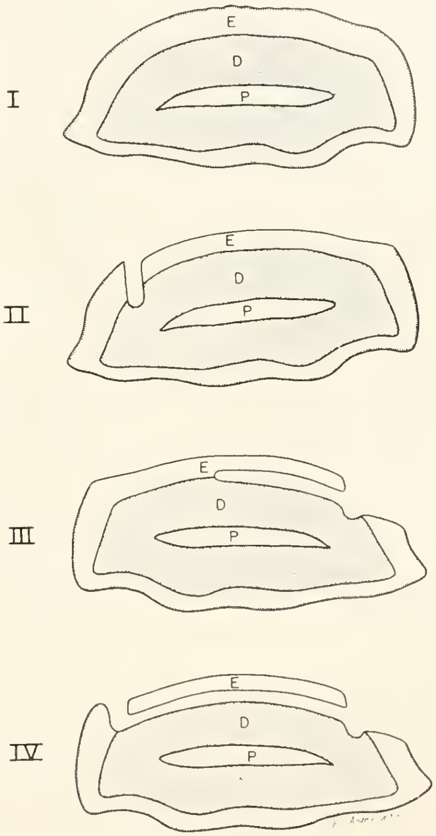


FIGURE 7. Schematic diagram of the dissection of the enamel as viewed through a stereo microscope. I. Appearance of the 1600-micron-thick cross-section of the crown of a bovine incisor tooth. Outer layer of cementum is shown by the short black lines at the surface. E = enamel; D = dentin; P = pulp space. II. The layer of cementum and the outer one-fourth of the enamel have been removed. III. Section turned over and dissection started at the dentino-enamel junction. IV. Slab of enamel completely dissected.

The gas source is a cylinder of nitrogen gas. Carbon dioxide, or a line supply of compressed air, if available at the required pressure, 80–100 psi, may also be used as the propellant gas.

Methods

To illustrate the use of the apparatus, the microdissection of the fully calcified enamel of erupted mature bovine incisor teeth will be described in some detail. Serial cross-sections of the crowns of 18- to 36-month-old steers are cut at about 1600 microns, using a modification of the Gillings-Hamco thin sectioning machine (Friberg and Levine, unpublished data). Because of the curvature of the tooth crown, it is not possible to obtain sections which are consistently at right angles to the surface. To compensate for this, the cross-section is first placed with the outer layer of cementum which covers the enamel sloping downward and outward (Fig. 6, I). The outer one-third to one-fourth of the enamel is then removed by the air-abrasive stream by cutting longitudinally from right to left, terminating with a right angle cut down into the dentin (Fig. 7, II). The section is next turned over so that the slope of the dentino-enamel junction is downward-inward (Fig. 6, II). A second longitudinal cut is made along the dentino-enamel junction, again from right to left, sacrificing approximately the inner one-fourth to one-third of the enamel and a corresponding width of the dentin (Figs. 6 II, 7 III). Finally, a right-angle end cut is made up through the enamel, isolating a slab of enamel, corresponding roughly to the middle one-half to one-third of the original enamel layer (Figs. 6 III, 7 IV, 8). Relatively large samples (50 gm.) of enamel were readily

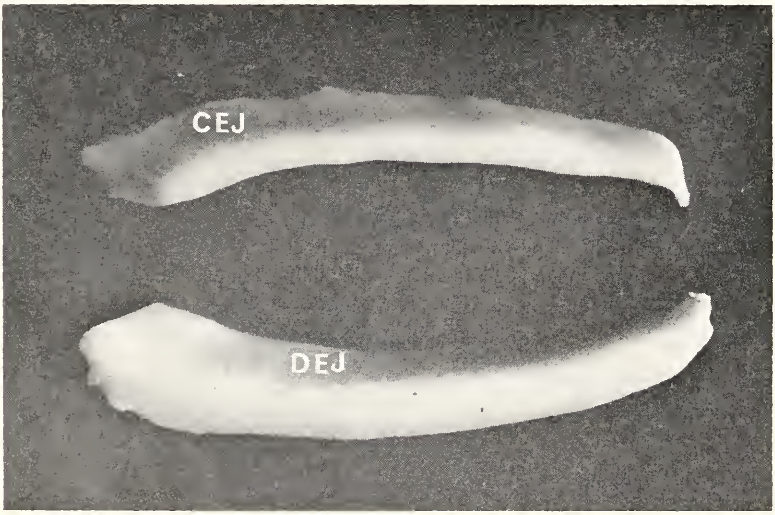


FIGURE 8. Slabs of enamel dissected by air-abrasive technique. DEJ = Enamel surface cut proximal to the dentino-enamel junction; CEJ = Enamel surface cut distal to the cemento-enamel junction. × 8.5.

prepared by this method from the labial side of the coronal surface only, since the enamel covering the lingual surface was too thin.

A second application of this technique has been the microdissection of the prismatic and nacreous layers of the shell of the mollusc, *Mercenaria mercenaria.* In this instance, however, because of the size and shape of the shell, it was difficult to cut the 1600-micron cross-sections of the shell in the Gillings-Hamco apparatus. These were readily prepared, however, by air-abrasive dissection using the 0.006″ × 0.060″ rectangular nozzle (Fig. 9). In a fashion similar to that described for enamel, the periostracum and the outer one-fourth of the prismatic layer were first removed from cross-sections of the shell, following which a slab of pure prismatic layer was dissected by air-abrasive cutting along the junction of the prismatic and nacreous layers (Fig. 10). The nacreous layer can be similarly isolated.

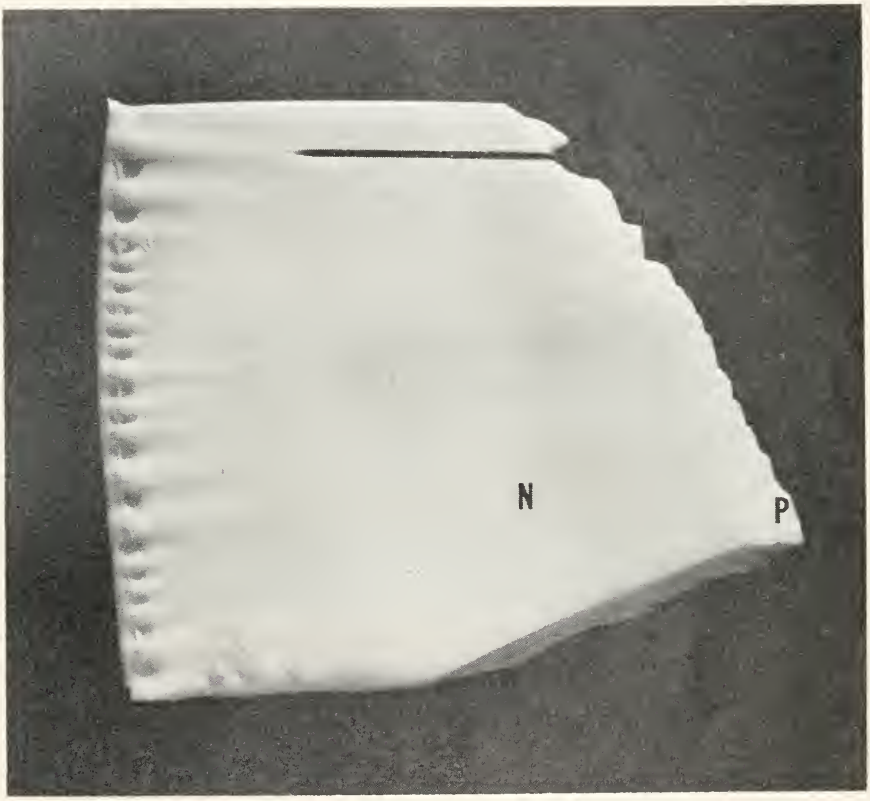


FIGURE 9. Lip portion of shell of *Mercenaria mercenaria* from which a slab has been partially cut, using a rectangular nozzle (0.006″ × 0.060″). This is to be used for the dissection of the prismatic layer. × 8.5. N = nacreous layer; P = prismatic layer.

The microdissection technique has also been used in the preparation of oriented blocks of mineralized material for electron microscopy and x-ray diffraction studies. For example, 300- to 500-micron-thick coronal or cross-sections of osmium-fixed, methacrylate- or epoxy resin-embedded teeth, were cut into strips 500 microns wide by air-abrasive microdissection. From such strips, segments of appropriate length from known locations and orientations can easily be cut. The segments are then re-embedded and sectioned on an ultramicrotome for use in electron microscopy. For x-ray diffraction studies, strips, slabs, rods, cubes, etc. of known location and orientation may be dissected directly from the tissue. Since the optimum thickness of mineralized tissues for x-ray diffraction is often less than 100 microns, further dissection by the air-abrasive method or hand grinding may be necessary.

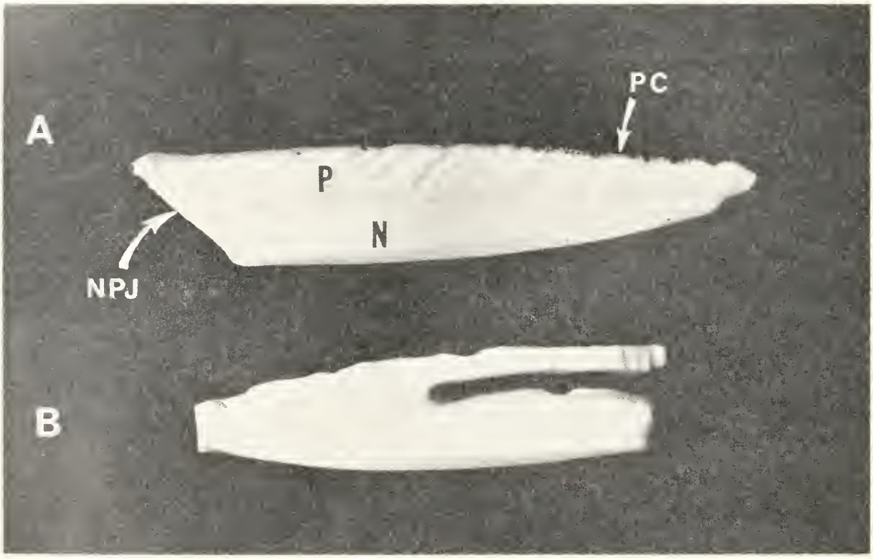


FIGURE 10. Preparation of a slab of prismatic layer from section prepared as in Figure 9. (A) original section; (B) after removal of the periostracum, a slab of the prismatic layer is being dissected by sectioning along the junction between the prismatic and nacreous layers. × 8.5. P = prismatic layer; N = nacreous layer; PC = periostracum and surface contaminants; NPJ = nacreous-prismatic junction.

DISCUSSION

Histologically defined tissue layers have been prepared from fully mineralized samples of adult bovine teeth and other highly mineralized structures, such as molluscan shells, by microdissection without prior chemical modification, utilizing a method which employs high speed, gas-impelled, abrasive particles to section the tissue. In the case of the molluscan shells, air-abrasive sectioning can also be utilized for the preliminary preparation of the cross-sections of the shells from which the individual layers are to be dissected. The air-abrasive method for the initial preparation is actually preferable to the conventional method utilizing a rotating disc (Gillings-Hamco or similar devices), in that it eliminates heating, wetting, and shattering of the sample.

Since the air-abrasive stream spreads from the orifice in conical fashion it is usually best not to exceed a section thickness of 2 mm. in order to maintain precision in microdissection; 1.6 mm. was found to be optimal for the enamel work. However, when the apparatus is used to obtain the initial cross-sections, a thickness of 3 mm. to 4 mm. can be tolerated. In the microdissection of blocks for electron microscopy or x-ray diffraction, sections of 500 microns or less can be handled if supported by a glass slide during cutting. For thin sections it is also advisable to reduce air pressure and abrasive feed and to increase cutting speed. The use of abrasive particles of much smaller size in combination with smaller orifice nozzles is under investigation for use in the preparation of smaller and thinner samples for micro-x-ray diffraction.

For precise work, it is essential that the specimen stage be motor-driven to provide slow, steady motion in the cutting direction during microdissection. The operator is thereby able to concentrate on tracing the respective tissue layer margins. In its present version, the apparatus described provides motor drive for the stage in one direction only, *viz.*, right to left, or reverse. Manual control of the cross-feed was retained since it was found to be adequate for the cutting of rectangular or slightly curved strips. In the preparation of bovine enamel, the surface cemental layer and the dentino-enamel junction of a section can readily be followed by small movements of the cross-feed crank wheel. The rotary feed is not used in the preparation of the enamel strips, but is of value in tracing more strongly curved lines. It would be advantageous for the routine preparation of circular or markedly curved structures also to motorize the cross-feed of the table, and to adopt a "joy-stick" control of the two motors, so that constant vectorial speed can be maintained during the microdissection.

The authors are indebted to Mr. O. Fontain for help with the mechanical construction of the apparatus, and to Mr. R. Cavicchi for assistance with the electrical circuitry.

SUMMARY

A method for the microdissection of mineralized tissues is described, utilizing high speed, gas-impelled abrasive for the sectioning. The method was developed to make possible the preparation of histologically defined samples of such tissues for biochemical and biophysical analysis. Mineralized tissue sections are mounted on a motorized, cross-feed stage and passed below a fixed nozzle delivering the abrasive stream. The operator controls the direction and progress of the microdissection through a stereo microscope. This procedure may be readily adapted to a wide variety of preparatory tasks involved in the study of mineralized tissues.

LITERATURE CITED

Glimcher, M. J., U. A. Friberg and P. T. Levine, 1963. The identification and characterization of a calcified layer of coronal cementum in erupted bovine teeth. *J. Ultrastruct. Res.*, in press.

Stucker, G. F., 1961. Salvaging fossils by jet. *Curator,* **4**: 332.

GENETIC INFLUENCE ON PHOTOTAXIS IN DROSOPHILA MELANOGASTER

NORTIN M. HADLER

Department of Biology, Yale University, New Haven, Connecticut

Historically, the behavior of many taxonomic groups of organisms has been treated as invariant. That individual differences were present was realized, but these variations were often dismissed as insignificant deviations from the behavior norm characteristic of the particular organism. Recent research, such as that of Hirsch (1959), Lewontin (1959), Erlenmeyer-Kimling and Hirsch (1961) and others, has demonstrated the inadequacy of this viewpoint. A more complete understanding of the behaving organism requires knowledge of individual differences in the population.

Drosophila melanogaster was the organism chosen by earlier workers for investigation of individual differences and analysis of the genetic contribution to observed behavior. This organism is readily available, has a short generation time, and is quite amenable to genetic analyses. Furthermore, strong geo- and phototaxes can be readily elicited for behavioral and genetic analysis. Hirsch and Tyron (1956) described a reliable technique for assessing the geotactic response of large numbers of individuals of *Drosophila melanogaster*. Hirsch and Boudreau (1958) later applied this technique in studying the heritability of phototaxis in *Drosophila melanogaster*. In this experiment a population of *Drosophila melanogaster* was screened by exposing each individual to a light-dark choice as it passed through a Y-tube. Each individual was tested ten times. Selection pressure was applied over 29 generations through assortative mating to produce strains varying greatly in their characteristic degree of positive phototaxis.

In the present paper a Y-maze for the study of phototaxis in *Drosophila* is described, with which large numbers of flies can be scored with high reliability. Animals passing through this apparatus make 15 successive light/dark choices, and their point of emergence is a measure of the strength of their phototactic response. In addition, two selection experiments are described, and their implications for the problem of phototaxis in *Drosophila melanogaster* and of the analysis of behavior in *Drosophila* in general are discussed.

METHOD

Apparatus

In 1959 Hirsch described a "multiple unit classification maze" for the mass screening of *Drosophila melanogaster* for geotaxis. I have constructed and used for two years analogous mazes for the study of phototaxis in *Drosophila melanogaster*. The photomaze consists of 15 consecutive Y-units. A population of 200 females and 200 males is introduced into the stem of the first Y, and in passing through the maze each individual makes 15 consecutive light/dark choices. The animals emerge in 16 collecting tubes, each containing a plug of culture medium. The collecting tube into which a fly emerges establishes how many light or dark choices it has made in passing through the maze. A "Plexiglas" cone (Hirsch, 1959) is inserted in each arm of a Y-unit to minimize re-tracing. A maze of N units has $N(N+1)/2$ Y-units, $N(N+1)$ cones and $(N+1)$ collecting tubes.

The structural unit of the maze is a black nylon (rubber in the case of Maze I) hexagon, $\frac{3}{4}''$ on a side and $\frac{5}{16}''$ in thickness. These are glued, sides parallel, onto a sheet of black "Plexiglas" so as to create alleys $\frac{5}{16}''$ wide and form a pattern of Y-units (Fig. 1). The cones are glued into position in the arms of each Y—a black "Plexiglas" cone in each "dark" arm and a clear cone in each "light" arm. A sheet of $\frac{1}{8}''$ "Plexiglas" is fastened over the hexagons by screws, forming a roof over the alleys. The sheet is painted black except over the "light" arm of each Y. The maze is screwed to a blackened sheet of plywood for support, and a circular fluorescent lighting fixture, 12″ in diameter, is suspended 26″ above the horizontal surface. Caution must be exercised in painting and glueing so as not to create a bias of odor or surface texture to compete with the light source as the differential stimulus. For additional information see Hirsch (1959).

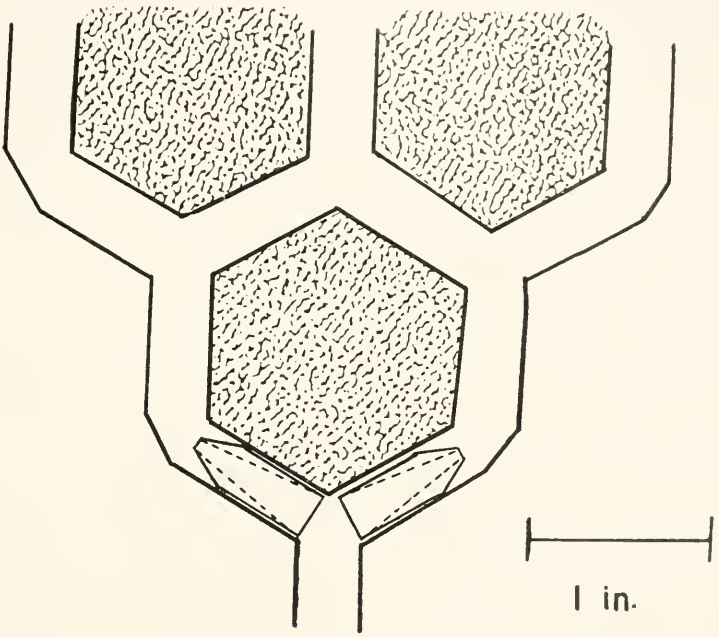


FIGURE 1. Diagramatic representation of the hexagonal structural unit of the photomazes. Alleys are formed by glueing black nylon hexagons onto a sheet of lucite. Lucite cones are inserted in each arm of a Y as shown.

Through qualitative observation it appears that mechanical stimulation, rearing conditions, age, temperature and humidity, light, and the effects of gravity must be maintained constant. To control mechanical stimulation while introducing the

TABLE I

Selection data

	Maze I				Maze II			
	Light		Dark		Light		Dark	
G1	♀	♂	♀	♂	♀	♂	♀	♂
$\bar{x}$	6.58	6.84	7.62	8.42	7.92	7.83	8.18	8.99
σ^2	5.35	7.79	4.95	4.94	7.83	7.94	6.16	7.73
G2								
$\bar{x}$	6.56	7.45	8.62	9.51	7.60	8.53	9.57	10.34
σ^2	4.04	5.37	4.88	5.21	6.91	7.79	5.67	7.32
G3								
$\bar{x}$	5.75	5.48	8.53	9.32	6.73	7.75	10.30	10.15
σ^2	7.03	5.37	6.09	6.00	7.26	6.68	4.85	5.54
G4								
$\bar{x}$	6.14	6.92	9.15	9.49	7.56	8.68	10.45	11.49
σ^2	5.28	5.14	6.82	7.85	7.26	7.76	6.36	5.46
G5								
$\bar{x}$	5.34	6.30	8.48	9.84	7.02	8.29	10.54	11.29
σ^2	11.06	8.14	13.19	7.04	8.73	6.80	8.45	6.11
G6								
$\bar{x}$	5.66	4.75	8.77	8.59	5.24	6.02	9.19	9.33
σ^2	5.42	5.54	6.06	9.26	6.14	7.08	4.54	7.60
G7								
$\bar{x}$	4.88	4.49	10.02	10.06	4.70	5.66	8.85	9.66
σ^2	5.10	7.01	5.63	9.94	5.13	5.90	6.16	7.87
G8								
$\bar{x}$	4.82	5.00	10.10	10.07	6.21	6.62	9.66	10.17
σ^2	5.42	7.22	7.03	7.59	7.40	8.26	6.98	6.30
G9								
$\bar{x}$	3.73	3.42	10.65	10.86	4.35	4.68	9.33	9.25
σ^2	4.44	4.87	4.89	6.59	4.07	4.76	6.65	7.72
G10								
$\bar{x}$	4.40	3.96	10.48	10.15	4.12	4.36	8.78	9.88
σ^2	6.45	4.87	5.41	6.29	5.22	4.97	8.38	7.38
G11								
$\bar{x}$	5.62	5.26	12.27	12.52	5.61	6.67	11.22	12.47
σ^2	5.11	5.27	4.08	5.17	5.92	5.38	5.20	5.51
G12								
$\bar{x}$	4.31	4.09	11.24	11.88	4.86	5.30	10.77	11.11
σ^2	5.08	4.59	6.63	4.58	5.73	6.31	4.70	6.76
G13								
$\bar{x}$	3.25	3.72	11.29	12.20	5.13	6.41	11.69	11.64
σ^2	5.49	4.47	5.02	4.05	8.50	8.49	7.93	5.88
G14								
$\bar{x}$	3.52	3.62	11.81	12.76	4.29	4.66	9.72	10.37
σ^2	3.83	4.86	4.13	2.57	6.27	5.54	7.25	6.44
G15								
$\bar{x}$	3.90	4.00	12.60	12.71	3.73	3.79	11.62	12.01
σ^2	3.99	5.47	4.70	4.54	5.10	5.33	5.75	4.45

Wildtype Controls

	Maze I		Maze II	
	♀	♂	♀	♂
$\bar{x}$	7.88 ± .58	8.70 ± .63	9.97 ± .52	10.46 ± .48
σ^2	6.03 ± 1.31	6.55 ± .99	7.72 ± 1.53	6.63 ± .54

animals into the apparatus, a sliding door blocks immediate access to the maze when the starting tube containing the flies is first attached. After sufficient time for the effects of mechanical stimulation, which accompany transfer to the maze, to abate (usually 30 minutes), the door to the maze is opened carefully to avoid agitation.

Two mazes have been constructed in this design, and separate selection experiments are being done with each maze. To increase the differences between the mazes, a double circular fluorescent fixture illuminates the surface of Maze II at about 300 apparent foot candles. Over Maze I, a single bulb fixture is suspended. In both mazes the animals are scored 0–15, corresponding to the numbers of the collecting tubes. Tube 0 receives those subjects which have made 15 consecutive light choices, *i.e.*, from whom the extreme measurable photopositive response has been elicited. Similarly, Tube 15 receives the most photonegative flies.

Subjects and procedure

The selection experiments described in this paper involved over 20,000 flies. The foundation population from which both dark and both light strains were derived was established in a population cage from equal numbers of Formosa, Capetown and Syosset strains of *Drosophila melanogaster* provided through the generosity of Prof. Th. Dobzhansky. Flies from this wild type population were passed through the maze, and selection was begun by mating 60 females and 60 males from the photopositive end of the distribution. Similar matings were done with flies at the photonegative end of the distribution. By this procedure photopositive and photonegative strains were established for each maze. In succeeding generations the extreme 60 males and 60 females from each strain were mated. Maze trials were 24 hours in duration, each beginning at approximately 6 PM to control for diurnal rhythms in behavior. The age of the 200 males and 200 females (run simultaneously) at the time of testing did not exceed 96 hours. Cultures were maintained at room temperature and humidity. The culture medium used in the Yale Laboratories is prepared with the following ratio of ingredients: 56.5 cc. H_2O/0.5 g. agar/6 cc. molasses/4.9 cc. cornmeal/0.7 g. brewers yeast/0.75 cc. 10% tegosept solution.

RESULTS

For fifteen generations selection pressure has been applied to produce highly photopositive ("light") and photonegative ("dark") strains. The results of these trials are given in Table I. Figure 2 shows the phototactic response of each strain as a function of generation number.

Included in Table I are the results of 9 wild type control populations tested with Maze I and 9 tested with Maze II. Presented are the averages of the means of these trials, averages of the variances and their respective standard errors. The number of flies in each trial was approximately constant. Note that because of a difference in stimulus environment, the flies of Maze II were characteristically more photonegative than those of Maze I. Even more interesting is the fact that the variances of the female populations tested in the two mazes are significantly different. However, when a single population was subjected to two consecutive trials in the same maze, the differences in variances were not significant (Table II).

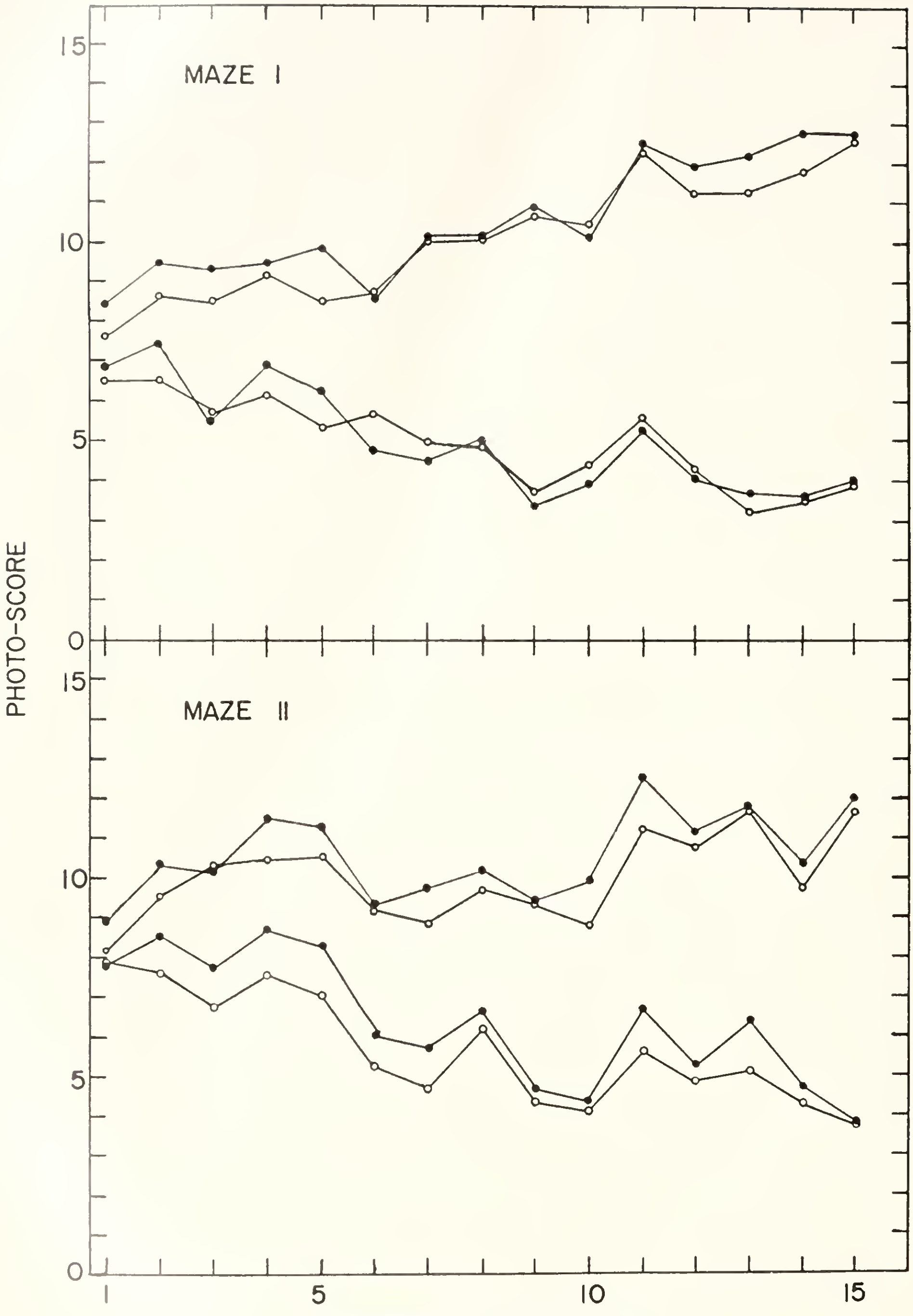

MAZE I
MAZE II
PHOTO-SCORE
0
5
10
15
1
5
10
15
GENERATION OF SELECTION

Figure 2.

TABLE II

Rerun data on wild type populations

			n_1	n_2	F	$Pr[t > \|t\|]$
A.	MI	♀	159	151	1.00	0.871
		♂	164	142	1.22	0.997
	MII	♀	190	171	1.02	0.997
		♂	180	180	1.15	0.936
B.	MI	♀	187	35	1.08	0.887
		♂	168	22	1.38	0.781
	MII	♀	209	51	1.24	0.997
		♂	221	43	1.19	0.875

A—rerun of entire population.
B—rerun of modal collecting tube population.
n_1—degrees of freedom of greater mean square.
n_2—degrees of freedom of lesser mean square.
N.B. all F values accept at the 5% level.

This suggests that Maze II elicits a greater variation in behavior in females than does Maze I and that this variation is purely a function of a genotype-environment interaction. After 15 generations of selection, however, neither the means nor the variances in the female population in Maze I were significantly different from those in Maze II. Thus, selection has effected a change in the genetic constitutions of the Maze I and Maze II populations so that the two mazes no longer represent significantly different stimulus environments. A difference in the variances of the flies tested in Maze I and Maze II was not observed in the male populations.

When the variance of the selected population approaches the standard error of the variance of the wild type population, selection will reach a limit. Thus the variances of the selected populations will approach the following asymptotes: Maze I Light Strain female 1.31, Maze I Light Strain male 0.99, Maze II Light Strain female 1.53, Maze II Light Strain male 0.54, and 15 minus these values for the corresponding dark lines. Until these asymptotes are closely approached no reliable estimate of heritability can be made.

From Figure 2 it is obvious that response to selection was immediate and quite strong. The variances of the later selected generations are highly significantly different from those of the unselected foundation populations. This is experimental evidence for the change in the genetic constitution of the population effected by selection in the photomazes. However, rather than compare the means of the selected and unselected populations, information as to the strength of selection can

FIGURE 2. Selection in Maze I and Maze II over 15 generations. The mean of the distribution of each sex of each strain is plotted as a function of generation of selection. The units of "photoscore" correspond to the numbers of the collecting tubes where the flies leave the maze. Flies emerging into Tube 0 have completed 15 consecutive light choices; into Tube 1, 14 light choices and one dark choice. Therefore, flies emerging into Tube 15 have completed 15 consecutive dark choices. Open circles represent females; closed circles, males.

TABLE III

t Tests with wild type and theoretical extreme means

Generation of selection	Sex	Strain	t	$Pr[t > \|t\|]$	d	Hypothesis
G13	♀	M_ILt	1.39	0.168	138	0
			−1.98	0.049	138	7.90*
	♂		1.76	0.081	133	0
			−2.35	0.020	133	8.70*
	♀	M_IDk	−1.65	0.100	189	15.00
			1.52	0.131	189	7.90
	♂		−1.39	0.165	203	15.00
			1.74	0.084	203	8.70
	♀	M_{II}Lt	1.76	0.080	166	0
			−1.67	0.097	166	10.00
	♂		2.20	0.029	160	0*
			−1.40	0.162	160	10.50
	♀	M_{II}Dk	−1.18	0.242	135	15.00
			0.60	0.550	135	10.00
	♂		−1.39	0.168	147	15.00
			0.47	0.039	147	10.50
G14	♀	M_ILt	1.80	0.074	195	0
			−2.24	0.026	195	7.90*
	♂		1.64	0.103	177	0
			−2.30	0.022	177	8.70*
	♀	M_IDk	−1.57	0.118	193	15.00
			1.92	0.056	193	7.90
	♂		−1.40	0.164	173	15.00
			2.53	0.012	173	8.70*
	♀	M_{II}Lt	1.71	0.088	236	0
			−2.28	0.023	236	10.00*
	♂		1.98	0.049	199	0*
			−2.48	0.014	199	10.50*
	♀	M_{II}Dk	−1.96	0.051	254	15.00
			0.10	0.917	254	10.00
	♂		−1.83	0.069	198	15.00
			−0.05	0.958	198	10.50
G15	♀	M_ILt	1.95	0.052	164	0
			−2.00	0.047	164	7.90*
	♂		1.71	0.089	168	0
			−2.01	0.046	168	8.70*
	♀	M_IDk	−1.11	0.270	203	15.00
			2.17	0.031	203	7.90
	♂		−1.08	0.284	171	15.00
			1.88	0.062	171	8.70
	♀	M_{II}Lt	1.65	0.100	214	0
			−2.78	0.006	214	10.00*
	♂		1.64	0.102	196	0
			−2.91	0.004	196	10.50*
	♀	M_{II}Dk	−1.41	0.161	201	15.00
			6.68	0.499	201	10.00
	♂		−1.41	0.158	190	15.00
			0.72	0.475	190	10.50

N.B. "*d*" is number of degrees of freedom, *i.e.*, number of individuals in population−1.
*t Test rejects at 5% level.

be obtained by comparing the means of the selected population with model populations bearing either a mean of 0, 15, or the mean characteristic of the wild type population for a particular maze and sex (from Table I). The results of these t tests for the later generations of selection are presented in Table III. In the G15 populations the following lines have diverged significantly from the appropriate wild type mean and are not significantly different from the appropriate extreme mean:

Maze I, "light" males and females
Maze I, "dark" females
Maze II, "light" males and females

The response to selection was apparently less strong for the dark lines than for the light lines. This can be accounted for in part by the fact that the wild type means, especially for Maze II, were in the photonegative half of the photoscoring range, *i.e.,* 7.5. There was therefore less room for screening and selection to operate in the dark side of the photomazes.

Discussion

"Taxis" is defined as "locomotory movement of an organism . . ., in response to a directional stimulus, the direction of movement being oriented in relation to the stimulus" (Abercrombie *et al.*, 1962). The crucial word is "oriented."

Three different experimental designs have been utilized in studying phototaxis in *Drosophila melanogaster:* (1) The rate at which flies approach a light source at the far end of a tube is measured (Carpenter, 1905; Payne, 1911; McEwen, 1918; Scott, 1937, 1943). (2) The distribution of flies in a field with a directed light source is recorded after a specified period (Carpenter, 1905; Lutz and Grisewood, 1934; Fardon *et al.*, 1937; Barigozzi and Tonissi, 1946; Dürrwachter, 1957; Wolken *et al.*, 1957). (3) The flies pass through a Y-tube and the number of animals entering each arm is determined (Brown and Hall, 1936; Fingerman, 1952; Hirsch and Boudreau, 1958). Although the term phototaxis has been used in describing all three of these experimental designs, it is quite obvious these procedures do not measure the same response. The first method confounds phototaxis with photokinesis. That there is a difference between methods (2) and (3) may be less obvious, but it is nevertheless quite real. For example, McEwen (1918) by measuring the spatial distribution in response to directed light source found the tan mutant of *Drosophila melanogaster* to be "negatively phototactic." When screened through my photomazes, a population of tan mutant has a mean performance characteristically more photopositive than that of wild type. However, it must be kept in mind that it is likely that both culture conditions and, even more important, the genetic backgrounds of the mutant stocks differed between the present work and McEwen's. These factors could greatly influence the observed behavior. These remarks indicate one of the major difficulties in comparing results from different laboratories—confusion as to what kind of experimental apparatus is needed to measure phototaxis.

In addition to experimental design there are other factors which make difficult direct comparisons of published data. A review of the literature, coupled with personal observations, indicates some fourteen environmental or experimental variables that will affect, to some degree, the phototactic response of *Drosophila:* genetic background of the tested population, temperature during the test, time of day of the test, time since anaesthetic, rearing conditions, mechanical stimulation (Lewontin, 1959), time since feeding, energy and wave-length of light (*cf.* Goldsmith, 1961), state of dark adaptation, number of observations or trials per individual (Dürrwachter, 1957), age (Dürrwachter, 1957) and sex. A phototactic response is therefore a property of a particular stimulus environment, broadly defined. Only responses obtained in like environments can be compared.

Finally, phototaxis is a population concept. As shown by Hirsch (1959) and confirmed and extended in the present work, part of the variation in response observed with a population of flies is genetic in origin. Work is presently underway to elucidate both the physiological and genetic differences between the photopositive and photonegative strains.

The author gratefully acknowledges his indebtedness to Profs. D. F. Poulson, J. Hirsch and T. H. Goldsmith for valuable consultation and for providing laboratory space and facilities. The invaluable assistance of Steven Weller in computer analysis and statistical analysis is gratefully acknowledged. This work was supported by National Science Foundation Undergraduate Research Participation Awards administered through Yale University.

SUMMARY

1. The design and construction of two multiple Y-unit mazes are described, which will permit the assessment of the mean and variance of phototactic behavior in *Drosophila* populations.

2. Using maze performances as criteria, selection pressure has been applied for 15 generations. By this procedure highly photopositive and photonegative strains have been produced. The strength and limits of selection in the different mazes are established.

3. By an analysis of the behavior of the selected and unselected strains, the interaction of the environmental and genetic influences on phototactic behavior in *Drosophila melanogaster* is demonstrated.

4. The necessity of recognizing individual differences in populations of experimental animals and the importance of a controlled environment in the study of phototaxis are discussed, with particular reference to *Drosophila.*

LITERATURE CITED

Abercrombie, J. B., C. Hickman and M. Johnson, 1962. A Dictionary of Biology. Aldine Pub. Co., Chicago.

Barigozzi, C., and G. Tonissi, 1946. Ricerche preliminari sul fototropismo del mutanti di *Drosophila melanogaster* Meig. *Commentationes, Pontificia Academia Scientarum,* 10: 431–459.

Brown, F. A., Jr., and B. V. Hall, 1936. The directive influence of light upon *Drosophila melanogaster* Meig and some of its eye mutants. *J. Exp. Zool.,* 74: 205–220.

Carpenter, F., 1905. Reactions of the pomace fly to light, gravity, and mechanical stimulation. *Amer. Nat.,* 39: 157–171.

DÜRRWACHTER, G., 1957. Untersuchungen über Phototaxis und Geotaxis einiger *Drosophila*—Mutanten nach Aufzucht in verschiedenen Lichtbedingungen. *Zeitschr. Tierpsychologie*, **14**: 1–28.

ERLENMEYER-KIMLING, L., AND J. HIRSCH, 1961. Measurement of the relations between chromosomes and behavior. *Science*, **134**: 1068–1069.

FARDON, J. C., M. J. CARROLL AND W. A. SULLIVAN, 1937. Reflection spectra of eye pigment in *Drosophila melanogaster* and its mutants. *Stud. Instit. Div. Thomae*, **1**: 197–206.

FINGERMAN, M. J., 1952. The role of the eye-pigments of *Drosphila melanogaster* in photic orientation. *J. Exp. Zool.*, **120**: 131–164.

GOLDSMITH, T. H., 1961. The color vision of insects. *In:* Light and Life, W. D. McElroy and B. Glass, eds., The Johns Hopkins Press, Baltimore, pp. 771–794.

HIRSCH, J., 1959. Studies in experimental behavior genetics: II. Individual differences in geotaxis as a function of chromosome variations in synthesized *Drosphila* populations. *J. Comp. Physiol. Psychol.*, **52**: 304–308.

HIRSCH, J., AND J. C. BOUDREAU, 1958. Studies in experimental behavior genetics: I. The heritability of phototaxis in a population of *Drosphila melanogaster*. *J. Comp. Physiol. Psychol.*, **51**: 647–651.

HIRSCH, J., AND R. C. TRYON, 1956. Mass screening and reliable individual measurement in the experimental behavior genetics of lower organisms. *Psychol. Bull.*, **53**: 402–410.

LEWONTIN, R. C., 1959. On the anomalous response of *Drosophila pseudoobscura* to light. *Amer. Nat.*, **93**: 321–328.

LUTZ, F. E., AND E. N. GRISEWOOD, 1934. Reactions of *Drosophila* to 2537 Å radiation. *Amer. Mus. Novit.*, **706**: 1–7.

MCEWEN, R. S., 1918. The reactions to light and to gravity in *Drosophila* and its mutants. *J. Exp. Zool.*, **25**: 49–106.

PAYNE, F., 1911. *Drosophila ampelophila* Loew bred in the dark for 69 generations. *Biol. Bull.*, **21**: 297–301.

SCOTT, J. P., 1937. Inherited behavior in *Drosophila*. *Proc. Indiana Acad. Sci.*, **46**: 211–216.

SCOTT, J. P., 1943. Effects of single genes on the behavior of *Drosophila*. *Amer. Nat.*, **77**: 184–190.

WOLKIN, J., A. MELLON AND G. CONTIS, 1957. Photoreceptor structures, II. *Drosophila melanogaster*. *J. Exp. Zool.*, **134**: 383–410.

MARINE BIOLOGICAL LIBRARY WOODS HOLE, MASS.

RESPIRATORY METABOLISM IN A MARINE DINOFLAGELLATE[1]

PETER W. HOCHACHKA[2] AND JOHN M. TEAL

Dept. of Zoology, Duke University, Durham, N. C., and Woods Hole Oceanographic Institution, Woods Hole, Mass.

The discovery by Sweeney and Hastings (1958) that cell division can be readily phased in *Gonyaulax* has increased interest in marine dinoflagellates for the analysis of metabolism in dividing cells. However, with the exception of some general data on photosynthesis in *Gonyaulax* (Hastings *et al.*, 1961) and *Gymnodinium* (Moshkina, 1961) as well as on the excretion of substances (Wangersky and Guillard, 1960), very little is known about the energy metabolism of dinoflagellates. And perhaps as unfortunate, even less can be assumed, for it has become apparent in recent years that the way in which energy metabolism is patterned in different algal groups can be quite diverse. We report here some experiments on the respiratory metabolism of intact cells of *Gymnodinium*, a common marine dinoflagellate.

MATERIALS AND METHODS

Pure cultures of *Gymnodinium nelsoni* (clone GSBL) were obtained from Dr. Guillard of the Woods Hole Oceanographic Institution. These were cultured in an enriched sea water medium (see Guillard and Ryther, 1962). Growth was determined, by mounting 1-ml. samples of cells immobilized with potassium iodide in a Sedgewick-Rafter cell. Growth was under either continuous light (400 foot candles) or in an 18-hour light- alternating with a 6-hour dark-period. In the latter case, cell division was somewhat phased, up to five times as many cells dividing per unit time within the dark-period than in the light-period. During the light-period, less than 2% of the cells could be seen dividing at any one time; during the 6-hour dark-period, 10% were dividing. The mean generation time for cultures at 21° C. was 3–4 days. Cultures were used for respiratory studies at densities between 7000 and 30,000 cells/ml., equivalent to about 150–600 μg. dry weight/ml.

In their studies of blue-green algae, Kratz and Myers (1955) found that cells suspended in phosphate buffer, bicarbonate buffer, or in the original growth medium all had equal respiratory rates. Our cells behaved similarly and we therefore adopted the third and simplest technique. Oxygen uptake was assayed with a Teflon-covered oxygen electrode (Kanwisher, 1959), with the platinum held -1.25 V. to the reference electrode, and 0.5 N KOH electrolyte. The electrode reaction itself consumes a molecule of O_2 for every 4e- of current flow. For

---

[1] Contribution No. 1371 from Woods Hole Oceanographic Institution.

[2] Peter W. Hochachka worked under physiology training grant supported by NIH.

small closed systems, this effect might produce a significant error; with large respirometers, it can be ignored.

Samples of cells were drawn aseptically from growing cultures and transferred into vials of 22-ml. size. A small stirring bar was placed into each respirometer. Each vial was then stoppered with a rubber stopper into which was fitted an O_2 electrode and a 16-gauge hypodermic needle (see Hochachka *et al.*, 1962). In operation, the vials were held in a 21° C. regulated water bath and stirred magnetically. Substances and inhibitors were added in volumes 1/100th that of the respirometers through the hypodermic needle without interrupting stirring of the cell suspension. The suspension was centrifuged at the end of a run, the cells were washed with H_2O, then dried to constant weight for Q_{O_2} calculations.

RESULTS

A typical Q_{O_2} (dry weight) for cells in exponential phase of growth under continuous light was about 4.4 mm.^{3} O_2/mg./hour. In cultures grown under alternating light- and dark-periods, the respiratory rate fluctuated. Rate figures for cells in the dark-period were as low as 1.5 mm.^{3} O_2/mg./hour; three hours before the onset of the dark period, the cells had a Q_{O_2} of about 2.5.

Most of the compounds tested, over concentration ranges of 10^{-4} to $10^{-2}M$, produced no change or less than a 5% change in endogenous respiration. These included: glucose, fructose, galactose, glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, ribose, glyoxylate, glycocoll, serine, α-alanine, methionine, glutamine, cystine, oxidized glutathione, diphosphopyridine nucleotide (DPN), DPNH, Coenzyme A, EDTA, and diethyl dithiocarbamate (DEC).

Respiratory inhibition was obtained by the compounds listed in Table I. Variations from the averages given were usually less than 10%. The values are based on final rates after treatment. The concentrations given are those found maximally effective. Poisons of the glycolytic enzymes did not depress respiration as effectively as those of the Krebs cycle. Inhibition with malonate and fluoroacetate was rapid, a new respiratory rate being established within a few minutes after the poisons were applied to the cell suspension. This rate, once established, did not change with time. However, in cells grown in alternating light- and dark-periods, the effectiveness of these poisons varied. In Table II, malonate sensitivity is recorded as a function of the light-dark periods. The values again are based on final rates after malonate addition compared to control rates. Respiratory blockage was greatest at the beginning of the dark-period and in the middle of the light-period. Two alternative explanations for this finding are available: (1) changes in permeability or (2) changes related to cell division (Stern, 1959; Sorokin and Myers, 1957) which, it will be recalled, was phased, about 5 times as many cells dividing per unit time in the dark-period than in the light. The last alternative is unlikely, since the generation time of the culture was long enough (3.5 days) to yield at any one time populations with cells at all stages of maturation.

The inhibition of respiration produced by the addition of oxaloacetate, citrate, and α-ketoglutarate is not understood. Oxidation of dihydroxyacetone dimer,

TABLE I

The effect of various compounds upon the O_2 uptake of Gymnodinium, *recorded as per cent change from the endogenous rate*

Inhibitory compounds	Concentration	No. of tests	Per cent change
Iodoacetate	10^{-3} *M*	12	−10
Fluoride	2 × 10^{-3} *M*	2	0
Arsenite	3 × 10^{-3} *M*	3	−51
Malonate	10^{-3} *M*	7	−65
(succinate restoration)	6 × 10^{-2} *M*	7	15
Fluoroacetate	10^{-2} *M*	4	−87
Azide	10^{-3} *M*	2	−52
Cyanide	3 × 10^{-3} *M*	2	−35
Oxaloacetate	10^{-3} *M*	6	−75
Citrate	10^{-3} *M*	4	−65
α-ketoglutarate	10^{-3} *M*	3	−76
Stimulatory compounds			
Dihydroxyacetone dimer	2 × 10^{-2} *M*	4	34
Acetate	10^{-3} *M*	9	5
Succinate	10^{-3} *M*	10	15
Fumarate	10^{-3} *M*	4	5
Malate	10^{-3} *M*	4	5
Malate plus acetate, each at	10^{-3} *M*	4	5
β-alanine	2 × 10^{-2} *M*	2	18
Glutamate	2 × 10^{-2} *M*	2	21
Ascorbate	6 × 10^{-4} *M*	4	505
	1.2 × 10^{-3} *M*	1	600
	1.8 × 10^{-3} *M*	1	900
Reduced glutathione (GSH)	6 × 10^{-4} *M*	5	504
Cysteine	6 × 10^{-4} *M*	2	67

glutamate, α-alanine, acetate, fumarate, and malate presumably occurred *via* the Krebs cycle.

The striking stimulatory effect of ascorbic acid, reduced glutathione (GSH), and cysteine on the respiration of a marine alga has not been reported previously. Some typical examples of this effect are shown in Table I for cells growing under continuous light.

At physiological pH ascorbate does not normally autoxidize (Mapson, 1958).

TABLE II

Changing sensitivity of respiration to 10^{-3} M malonate during the light-dark cycle. Data expressed as per cent decrease from endogenous rates of O_2 uptake measured at the same time

Time in light period	No. of tests	% Decrease in O_2 uptake
6 hours	3	15
9	3	57
12	3	60
15	3	15
Time in dark period		
1–2 hours	3	94
3–4	5	31

In control respirometers containing only autoclaved sea water medium, we found no detectable autoxidation at low concentrations of ascorbate (10^{-5} to 10^{-4} M); at higher concentrations of ascorbate, a significant autoxidation occurred (Table III). So that corrections for autoxidation would be unnecessary in experiments with cell suspensions, concentrations of 10^{-4} M were used routinely. Over a concentration range of 10^{-5} to 10^{-2} M, cysteine and GSH did not autoxidize under our conditions.

TABLE III

The utilization of O_2 by autoxidation of ascorbate in an autoclaved sea water medium used for growing Gymnodinium, *expressed as mg. O_2 used per hour*

Test system	O_2 uptake
Sea water	0.01
Sea water + 6 × 10^{-4} M ascorbate	0.01
Sea water + 3 × 10^{-3} M ascorbate	0.04
Sea water + 5 × 10^{-2} M ascorbate	0.08

In other plants, a GSH-ascorbate electron transfer scheme is fairly well characterized (Mapson, 1958, for a recent review). Generally, arsenite and iodoacetate activate the oxidation of ascorbate to dehydroascorbate. DEC and cyanide inhibit ascorbic acid oxidase, but EDTA, GSH, and cysteine do not affect the purified enzyme from higher plants. The ascorbic acid oxidase is atypical in the slime molds in that cyanide and azide produce slight but definite activation (Ward, 1955).

With *Gymnodinium*, the compounds tested were found to affect ascorbate, GSH, and cysteine oxidations in a similar manner. The data are in Table IV.

In each case, several concentrations were tested. Only those at which active compounds were maximally effective are recorded in Table IV; the ranges of concentrations tested are indicated by compounds which did not seem to affect ascorbate oxidation. The effect produced by the active compounds was rapid and usually did not change over fairly short time periods.

As in higher plants, ascorbate oxidation in *Gymnodinium* is activated by arsenite but unaffected by iodoacetate. Cyanide enhanced ascorbate oxidation, as in the slime mold, but azide was without effect. Since the latter two compounds inhibited endogenous respiration, presumably by blocking the cytochrome chain, these findings rule out the possibility that ascorbate oxidation was catalyzed by cytochrome oxidase (Webster and Frenkel, 1953). The oxidation of ascorbate was also atypical in that it was unaffected by DEC, presumably because of lack of penetration.

Although EDTA did not affect respiration of control cell suspensions, it completely inhibited ascorbate oxidation. Again, this is not typical of the oxidase in other sources, and can be explained in two ways: either the oxidation may be proceeding by copper catalysis (Mapson, 1958) and not by a specific ascorbic acid oxidase; or secondly, since copper is known to be released during reaction of ascorbic acid oxidase, EDTA-inhibition may arise by chelation of this copper with concomitant inactivation of the enzyme. The action of GSH and cysteine on ascorbate oxidation might be explained in a similar manner (Mapson, 1958). By comparison, oxidized glutathione, cystine, and methionine did not affect ascorbate oxidation.

TABLE IV

The effect of various compounds on ascorbate, GSH, and cysteine oxidation in Gymnodinium, *expressed as per cent change. Numbers in parentheses refer to number of tests. Cells grown in continuous light*

Compound	Concentration	Percentage effect on oxidation of 10^{-4} M		
		Ascorbate	GSH	Cysteine
Malonate	10^{-3} M	−100 (3)	−100 (2)	−100 (2)
Fluoroacetate	10^{-3} M	−100 (3)	−100 (2)	−100 (2)
Iodoacetate	5×10^{-4} to 4×10^{-3} M	0 (5)		
Arsenite	2×10^{-3} M	+170 (3)		0 (2)
Azide	5×10^{-4} to 10^{-3} M	0 (3)	0 (2)	0 (2)
Cyanide	10^{-3} M	+ 40 (3)		
DEC	5×10^{-4} to 4×10^{-3} M	0 (3)		
EDTA	5×10^{-4} M	−100 (5)		
Cysteine	10^{-3} M	− 67 (2)		
GSH	10^{-3} M	− 75 (2)		− 94 (2)
Cystine	5×10^{-4} to 10^{-2} M	0 (2)	0 (2)	0 (2)
GSSG	5×10^{-4} to 10^{-2} M	0 (2)	0 (2)	
Methionine	5×10^{-4} to 10^{-2} M	0 (2)	0 (1)	0 (1)

The inhibition of the GSH-ascorbate path by malonate and fluoroacetate was also very pronounced, suggesting a coupling of such a chain to the Krebs cycle. This suggestion is in accord with Young and Conn (1956), who found that GSH oxidation occurred in mitochondria in the presence of catalytic amounts of ascorbic acid; reduction of oxidized glutathione was coupled to the oxidation of Krebs cycle intermediates. Though in *Gymnodinium* ascorbate oxidation seemed to be concentration-dependent (Table I), this likely was not due to a greater utilization of ascorbate *per se*. By calculation it can be shown that complete oxidation of 5×10^{-4} M ascorbate would deplete all available oxygen in our respirometers. As Figure 1 shows, this did not occur. Perhaps the concentration dependence of ascorbate oxidation is somehow related to a reaction-inactivation which we observed. A typical recording (Fig. 1) shows the pattern of decay in the ascorbate-stimulated respiration. This effect, which is also found in higher plants, may be due to lowered O_2 tensions, or to H_2O_2, which is thought to produce the reaction-inactivation in higher plants (Tokuyama and Dawson, 1962).

A shortage of time prevented any but brief analysis of GSH and cysteine oxidation. On the basis of the data in Table IV and the fairly well characterized system in higher plants, it is assumed that GSH oxidation is tied up with ascorbate metabolism. This, however, is in need of much further study. Perhaps it is worth mentioning that the reaction, cysteine + oxidized glutathione ⇌ GSH + cystine, can proceed spontaneously; hence, cysteine oxidation might simply represent GSH oxidation. Alternative explanations are available.

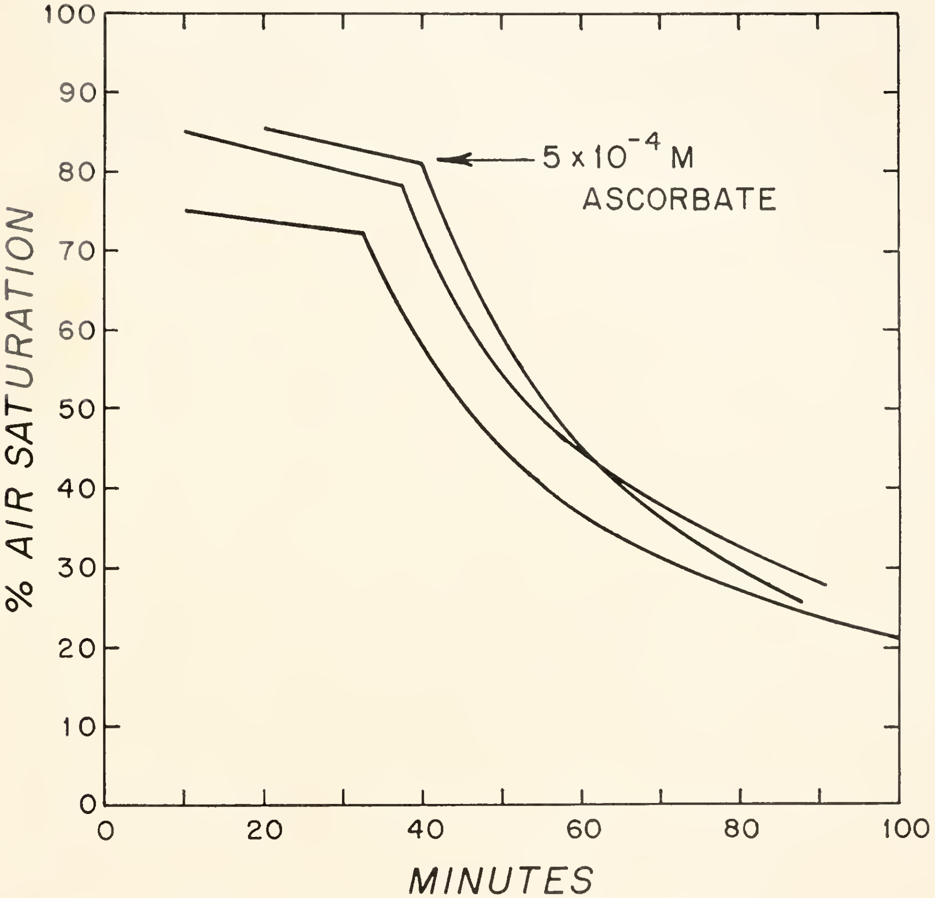


Figure 1. A typical recording of decreasing effect of ascorbate addition with time and oxygen concentration on metabolism of intact cells of *Gymnodinium nelsoni*. The experiment was done in triplicate. Rate of oxygen uptake is proportional to the slope of the lines.

From these pilot experiments, it is difficult to determine the contribution of a GSH-ascorbate system to the endogenous respiration of the organism. The large residual respiration remaining after azide and cyanide treatments of normal cells presumably occurs *via* an electron transport chain other than the cytochromes. Evidence of such a situation exists for other algae: *Anabaena*, for example, is insensitive to CO and sensitive to cyanide and azide only at high concentrations (Webster and Frenkel, 1953); cytochrome oxidase apparently is absent in some strains of *Chlorella* (Kolesnikov and Einor, 1961). However, if a portion of endogenous respiration in *Gymnodinium* depended upon a GSH-ascorbate electron transfer chain, one would expect an inhibitor to lower the endogenous rate and activating compounds to raise it. As Table IV shows, EDTA is an effective inhibitor while arsenite is an effective stimulator of ascorbate oxidation in these cells. But, as previously mentioned, EDTA has no effect on endogenous respiration and arsenite actually inhibits it. Therefore, there can be little if any participation of a GSH-ascorbate system in endogenous respiration of these algae. In higher plants, too, the full potential effect of ascorbic acid oxidase is not realized *in vivo*. In pea stem internodes, for instance, the enzyme is capable of consuming O_2 at a rate 40 times as great as the total respiration of the tissue (see Mapson, 1958). In algae, then, the physiological role of this system remains unclear.

SUMMARY

1. The effects of various inhibitors and substrates on the respiration of suspensions of *Gymnodinium* were tested. Iodoacetate, arsenite, malonate, fluoroacetate, azide and cyanide all inhibited the endogenous rate to some extent. Malonate and fluoroacetate were most potent. Cells grown in cycles of alternating light and dark were most sensitive to malonate during the middle of the light-period and at the beginning of the dark-period. Most of the glycolytic intermediates used did not affect the endogenous respiratory rate. Krebs cycle intermediates either increased the respiratory rate (succinate, malate, fumarate) or markedly decreased it (oxaloacetate, citrate, α-ketoglutarate). With the exception of an increased O_2 consumption in the presence of glutamate, alanine, and cysteine, the O_2 uptake of *Gymnodinium* was unaffected by the amino acids tested.

2. Ascorbate and reduced glutathione increased O_2 uptake profoundly. This uptake was further stimulated by cyanide and arsenite; it was insensitive to diethyldithiocarbamate and azide; it was completely blocked by cysteine, EDTA, malonate and fluoroacetate.

LITERATURE CITED

GUILLARD, R. R. L., AND J. H. RYTHER, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Canad. J. Microbiol.*, **8**: 229–239.

HASTINGS, J. W., L. ASTRACHAN AND B. SWEENEY, 1961. A persistent daily rhythm in photosynthesis. *J. Gen. Physiol.*, **45**: 69–76.

HOCHACHKA, P. W., J. M. TEAL AND M. TELFORD, 1962. Pathways of carbohydrate metabolism in lobster hepatopancreas. *Canad. J. Biochem. Physiol.*, **40**: 1042–1050.

KANWISHER, J., 1959. An oxygen electrode for biological use. *Limnol. Oceanography*, **4**: 210–217.

KOLESNIKOV, P. O. AND L. O. EINOR, 1961, 1962. A study of metal containing oxidases in *Chlorella* sp. *Ukrain. Bot. Zhur.*, **18**: 46–51. Referat *Zhur. Biol.*, **4**: V23.

KRATZ, W. A., AND J. MEYERS, 1955. Photosynthesis and respiration of three blue-green algae. *Plant Physiol.*, **30**: 275–282.

MAPSON, L. W., 1958. Metabolism of ascorbic acid in plants; Part I. Function. *Ann. Rev. Plant Physiol.*, **9**: 119–150.

MOSHKINA, L. V., 1961. Photosynthesis by dinoflagellatae of the Black Sea. *Fisiol. Rastenii*, **8**: 129–132.

SOROKIN, C., AND J. MEYERS, 1957. The course of respiration during the life cycle of *Chlorella* cells. *J. Gen. Physiol.*, **40**: 579–592.

STERN, H., 1958. Mitosis and metabolic organization. *Bot. Rev.*, **25**: 351–384.

SWEENEY, B., AND J. W. HASTINGS, 1958. Rhythmic cell division in populations of *Gonyaulax polyedra*. *J. Protozool.*, **5**: 217–224.

TOKUYAMA, K., AND C. R. DAWSON, 1962. On the reaction inactivation of ascorbic acid oxidase. *Biochem. Biophys. Acta*, **56**: 427–439.

WANGERSKY, P. J., AND R. R. L. GUILLARD, 1960. Low molecular weight organic base from the dinoflagellate *Amphidinium carteri*. *Nature*, **185**: 689–690.

WARD, J. M., 1955. The enzymatic oxidation of ascorbic acid in the slime mold, *Physarum polycephalum*. *Plant Physiol.*, **30**: 58–67.

WEBSTER, G. C., AND A. W. FRENKEL, 1953. Some respiratory characteristics of the blue-green alga, *Anabaena*. *Plant Physiol.*, **28**: 63–69.

YOUNG, L. C. T., AND E. E. CONN, 1956. The reduction and oxidation of glutathione by plant mitochondria. *Plant Physiol.*, **31**: 205–211.

UPTAKE, TURNOVER AND EXCRETION OF I-131 BY RAINBOW TROUT (SALMO GAIRDNERI)[1]

J. B. HUNN[2] AND P. O. FROMM

Department of Physiology and Pharmacology, Michigan State University, East Lansing, Michigan

One of the first observations on iodine metabolism in fishes was that of Marine and Lenhart (1910a, 1910b) who showed that addition of potassium iodide to water flowing past brook trout (*Salvelinus fontinalis*) could reduce the goitrous condition of these fish. Little additional information, exclusive of a few measurements on the iodine content of the thyroids of fish, was forthcoming until the advent of radioactive iodine. Since that time numerous studies have been made on iodine metabolism in fishes, with the bulk of the work being concentrated on thyroid physiology and biochemistry (see reviews by Pickford and Atz, 1957; Berg, Gorbman and Kobayashi, 1959; Leloup and Fontaine, 1960).

Studies of extra-thyroidal iodine have generally been limited to describing tissue other than the thyroid which concentrates iodine. Using injected doses of I-131, it has been established that the ovary of cyclostomes and teleosts, notochord of cyclostomes as well as gills and stomach of selachians will concentrate iodide (I-131) above the blood level. Maqsood, Reineke and Fromm (1961), using *in vitro* incubation, studied a number of tissues from rainbow trout and found that only the lower jaw (thyroid) concentrated I-131 above the level in the incubation medium. Few data, however, are available on the actual iodine content of nonthyroidal tissues exclusive of the blood. Robertson and Chaney (1953) reviewed the literature and have contributed values on the iodine content of a number of tissues of rainbow trout.

The level of injected radioiodine in the vascular compartment and its rate of turnover have been little studied in fish and concern blood levels and excretion rates. Leloup (1952) found that in mullet (*Mugil auratus*) the blood level of I-131 was 3.21% of the injected dose (per gram of blood) 8 hours after injection and 2.38% after 72 hours. Mullet pre-treated with thiourea (1 gram per liter of aquarium water) for 8 days had a blood level of 1.68% of the injected dose 8 hours after injection. Hickman (1959) reported the half-life of I-131 in the blood of salt-water flounder (*Platichthys stellatus*) ranged from 75 to 527 hours while that of the fresh-water flounder ranged from 203 to 279 hours.

Most studies of iodine excretion in fish have been concerned with determination of whole body loss; the findings showed great variation among different fishes. Leloup (1952) showed that mullet lose 24.7% of an injected dose of carrier-free I-131 in 24 hours and 45.3% in 72 hours; conger eels (*Conger*

---

[1] Published with the approval of the director, Michigan Agricultural Experiment Station, as Journal series No. 3203. This project was supported in part by the National Institutes of Health.

[2] Pre-doctoral NIH Research Fellow.

conger) lost 7% in 24 hours. Chavin (1956) found that goldfish (*Carassius auratus*) lost 65% of an injected dose in 24 hours, and Leloup and Fontaine (1960) indicate that rainbow trout at 20° C. lose 33.5% in 24 hours. It is of interest to note that Leloup (1952) found that mullet treated with thiourea excreted radioiodine at a greater rate than untreated controls and the blood level of I-131 remained below that of the controls.

The only information found on the routes and relative rates of excretion of iodide in fish is that obtained by E. K. Marshall, Jr. and published by Beiter (1933). In this study two aglomerular toadfish, *Opsanus tau*, were injected with NaI and after 23 hours 74% and 92% of the injected dose had been excreted by the gills while 0.67% and 1.1% was excreted by the kidney. Hickman (1959) has estimated that more than 80% of the I-131 injected into salt- and fresh-water starry flounder is excreted extrarenally.

Materials and Methods

Rainbow trout (*Salmo gairdneri*) weighing from 75 to 173 grams, provided by the Michigan Department of Conservation, Wolf Lake Hatchery, were kept under constant illumination in static tanks of aged, aerated tap water at 12 ± 2° C. They were fed trout pellets every other day and the water was changed every third day. During experimental periods the fish were not fed.

Ten trout used in the 24-hour uptake study were injected intraperitoneally with carrier-free Na I-131 made up in distilled water. The injection volume was 0.1 ml. and contained 2.26 μc. of I-131. Five fish were also given injections of 20 mg. of NaSCN. The fish were then placed in separate aquaria containing 36 liters of aerated, aged, tap water. After 24 hours the fish were anesthetized with MS-222 and samples of blood, upper gill and lower jaw were obtained. Gill and jaw samples were rinsed in fish Ringer's solution, blotted dry and weighed to the nearest milligram, using a Roller-Smith balance. All samples were wet-ashed with nitric acid in five-dram plastic vials and diluted to a counting volume. Counts were made on 10-ml. aliquots of the aquarium water. Counting standards were made up in similar vials using 1/20 of the injected dose. All samples were counted using a two-inch thallium-treated NaI well scintillation detector, Model PHA-ICA pulse height analyzer and Model DS-1A decade scaler used as a slave scaler. Counting was done at the 5% level of error or less.

For blood turnover studies, 15 trout were injected with 3.87 μc. Na I-131 per fish, then evenly distributed among three aquaria, each containing 38 liters of aged tap water and equipped with a charcoal filter. Five fish were killed at 24, 72 and 120 hours and samples of blood and lower jaw taken. Blood samples were centrifuged at 2500 rpm for 20 minutes and plasma samples varying from 0.4 to 0.7 ml. removed. Plasma proteins were precipitated with 10% $ZnSO_4$ and 0.5 N NaOH and washed twice with 10 ml. of glass-distilled water. Precipitates, washes, tissue samples and aquaria water were counted as noted above.

Routes of excretion of I-131 were studied in two groups of trout. The first group contained radioiodide that had been accumulated by the fish during a 24-hour exposure to distilled water containing Na I-131. The second group was studied 24 hours after receiving an intraperitoneal injection of Na I-131. For this experiment the fish were anesthetized with MS-222 and a polyethylene

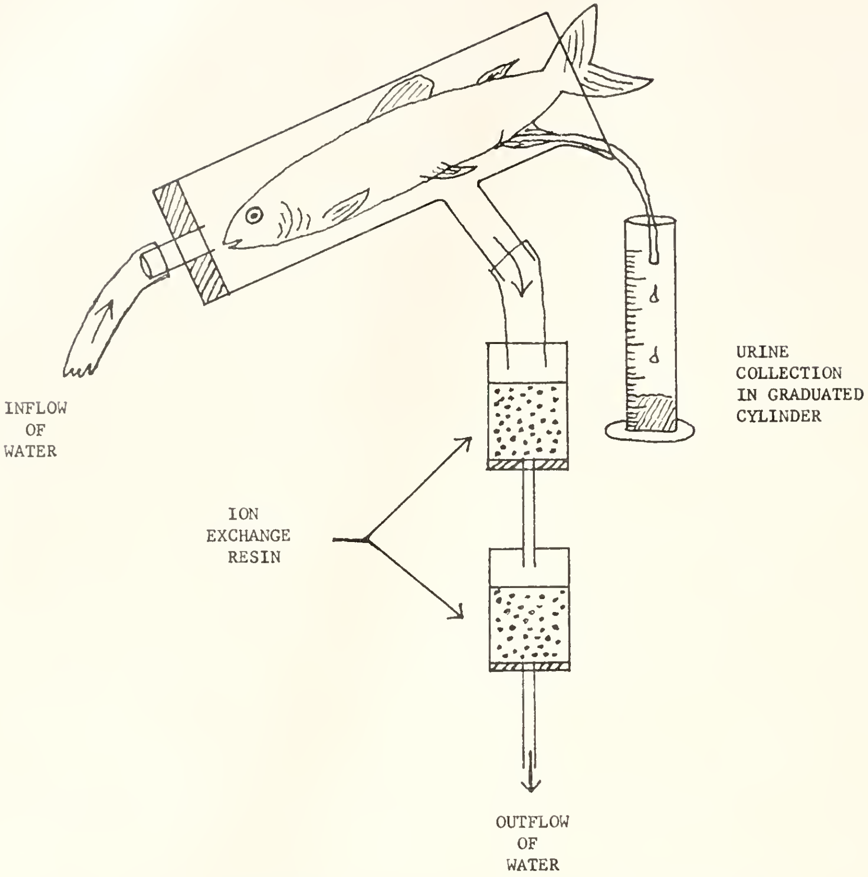


FIGURE 1. Distilled or tap water pumped over the gills of the experimental fish in the chamber, flowed out the side-arm into the two columns, each containing 10 grams of ion exchange resin, IRA 400. The cannula delivered urine into a 10-ml. graduate cylinder. To secure the animals in position damp cotton was tucked in around the tail of the fish.

cannula (Intramedic PE 60) with a flared tip was inserted into the urinary bladder. The fish was inserted into the urine-collecting apparatus (Fig. 1), damp cotton was placed around the posterior ends of the fish to keep them in place and the gills were bathed with tap or distilled water. At the end of the collection period the fish were again anesthetized with MS-222, blood samples taken, centrifuged and plasma drawn off. The radioactivity in the plasma, urine and resin samples was measured as noted above.

RESULTS

Data on the 24-hour tissue distribution of intraperitoneally injected Na I-131 (Table 1) indicate that the gills of trout do not accumulate iodide above the concentration found in the blood. In contrast, the lower jaw (thyroid) ex-

TABLE I

Twenty-four hour uptake of intraperitoneally injected I-131 by rainbow trout

Body weight $\bar{x}$ and range	n	% injected dose per gram tissue		
		Blood ($\bar{x}$ ± SE)	Gill ($\bar{x}$ ± SE)	Lower jaw ($\bar{x}$ ± SE)
Control				
129.7 (89.1–159.1)	5	3.10 ± 0.51	0.62 ± 0.06	11.20 ± 1.50
NaSCN (20 mg./fish)				
147.6 (98.1–172.6)	5	0.64 ± 0.16	0.26 ± 0.07	0.31 ± 0.07

hibits a definite facility for concentrating iodide which is inhibited by thiocyanate. In addition to blocking thyroidal uptake of radioiodine, administration of thiocyanate reduced the I-131 content of both blood and gill tissue. The I-131 content of the aquarium water 24 hours after injection showed that the rate of loss from the thiocyanate-treated fish was some three times greater than that of control fish (control 21.9% and NaSCN treated 69.7% of the injected dose).

Data on the relative distribution of I-131 in blood plasma following intraperitoneal injection are given in Table II. Less than 5% of the total radioiodide in the blood was found in the protein-bound fraction. The amount of protein-bound iodide decreased along with a decrease in the non-bound iodide until the fifth day after injection when it increased slightly, presumably the result of thyroidal production and/or release of radioactive thyroxine. Thyroidal (lower jaw) radioiodine showed a continual rise during the five-day experimental period.

A very short biological half-life of 1.7 days (Fig. 2) indicates a very rapid rate of turnover for non-bound I-131 in trout plasma. The rate of disapppearance of I-131 from the plasma is a function of tissue exchange (addition and withdrawal) and excretion (renal and extrarenal).

The apparatus, as shown in Figure 1, was constructed to facilitate the study of routes and rates of excretion of I-131 from trout. The urine flow rates

TABLE II

Distribution and turnover of I-131 in the blood plasma of rainbow trout

	24 hours $\bar{x}$ ± SE*	72 hours $\bar{x}$ ± SE*	120 hours $\bar{x}$ ± SE*
% dose per gm. plasma	4.53 ± 0.61	2.35 ± 0.41	0.91 ± 0.25
% dose non-bound per gm. plasma	4.49 ± 0.60	2.33 ± 0.41	0.88 ± 0.11
% dose protein-bound per gm. plasma	0.037 ± 0.004	0.016 ± 0.003	0.026 ± 0.003
% I-131 protein-bound	0.83 ± 0.05	0.70 ± 0.11	2.90 ± 0.13
% dose per gm. lower jaw	7.87 ± 1.38	18.94 ± 4.82	31.79 ± 6.78
$\frac{\text{Lower jaw I-131}}{\text{Blood I-131}}$	1.82	7.89	36.36

* n = 5.

(Table III) were calculated from data obtained during collection periods ranging from 75 to 120 minutes. Urine collected ranged in volume from 1.2 to 1.9 ml. The flow values obtained are high compared to the values for rainbow trout given by Krogh (1939), Holmes (1961) and Fromm (1963). Despite this, the urine/plasma ratios indicate that the trout kidney is quite efficient in reabsorbing iodide from the glomerular filtrate. The outflux of I-131 from the gills, measured simultaneously with urinary excretion, indicates that some 3.8 times more iodide is excreted by the kidney than by the gills per unit of time. Whether the urine/gill excretion ratio remains constant or changes with iodide levels in the plasma is not known.

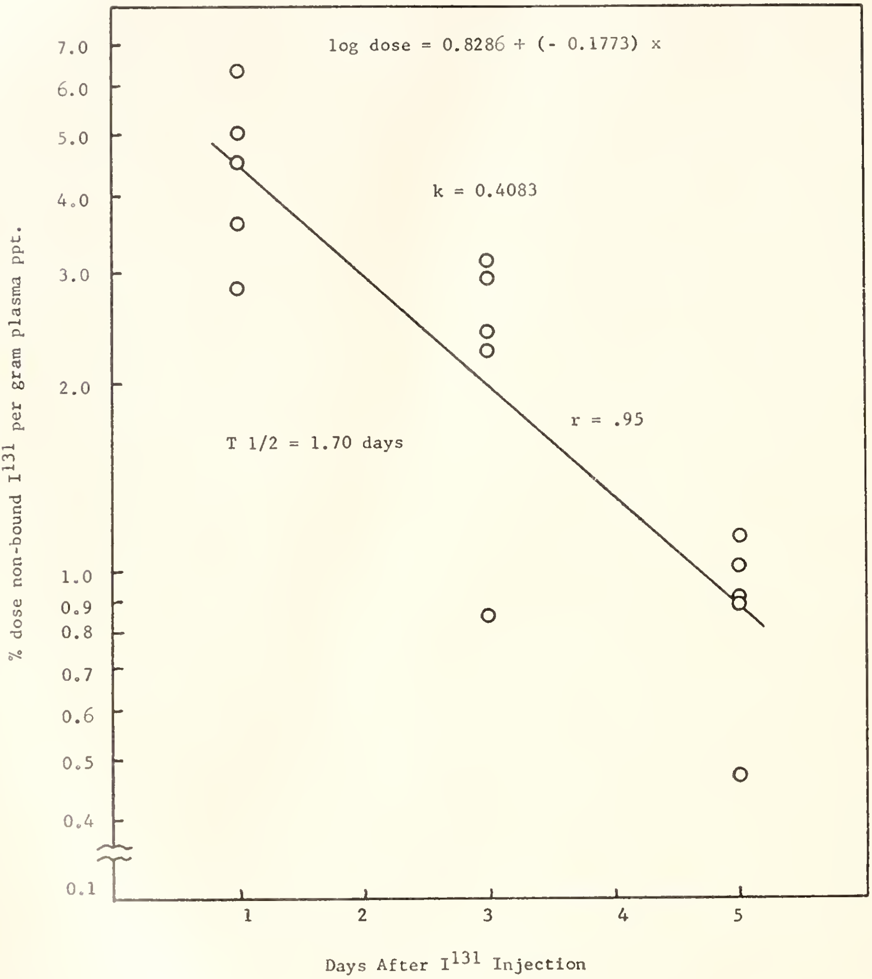


FIGURE 2. The biological half-life of non-bound I-131 in the blood plasma of rainbow trout.

TABLE III

Excretion of I-131 by rainbow trout

	Urine flow ml./Kg./day	I-131 urine/plasma ratio	I-131 clearance ml./hr.	I-131 urine/gill excretion ratio
Group I*				
1	129.6	0.30	0.22	3.92
2	232.2	0.07	0.06	2.74
3	160.8	0.27	0.17	6.29
4	211.2	0.34	0.25	3.05
5	190.0	0.10	0.10	3.61
Mean	181.0	0.22	0.16	3.92
Group II**				
1	176.4	0.08	0.04	4.0
2	151.2	0.09	0.06	4.5
3	153.6	0.10	0.06	—
4	192.0	0.07	0.04	2.66
Mean	168.3	0.08	0.05	3.72

* Group I exposed to I-131 in distilled water.

** Group II injected with I-131.

DISCUSSION

The effect of goitrogens on thyroidal uptake of I-131 in fishes is similar to that observed in mammals. The handling of the I-131 available due to blocking of the thyroid is, however, quite different. Bricker and Hlad (1955) have shown that administered SCN^- causes a marked increase in the plasma iodide I-131 levels in human hyperthyroid patients and a moderate rise in euthyroid patients. Similar increases in blood I-131 did not occur in trout treated with thiocyanate (Table I) and Leloup (1952) found no increase in blood I-131 of mullet and conger eels treated with thiourea and thiouracil, respectively. If the I-131 is not taken up by the thyroid and there is no increase in blood I-131, then the I-131 must be sequestered elsewhere or excreted.

I-131 levels in the aquarium water 24 hours after injection show that SCN^--treated trout lost three times as much I-131 as the controls. Leloup (1952) presented data showing that thiourea and thiouracil treatment of mullet and conger eels also increased excretion from two to eight times that of the controls. Bricker and Hlad (1955) have shown, however, that in humans SCN^- does not appreciably alter the I-131/inulin clearance ratios. If the fish kidney responds to SCN^- in a similar manner, then the increased excretion observed must have been extrarenal, possibly by way of the gills. The role of the gill in excreting halides (chloride) is well-established in marine fishes. Hickman (1959) and Leloup and Fontaine (1960) have shown that I-131 will accumulate in the gills at levels above that of the blood in both the starry flounder and dogfish (*Scyllium canicula*). This suggests that the gills may excrete iodide and this function is consistent with known chloride metabolism. Certainly the build-up of iodide in the gills could reverse the concentration gradient across the gills which would permit a passive loss of iodide from the fish to the environment. Both *in vitro* and *in vivo* studies have shown, however, that no build-up of iodide (I-131) occurs in the gills of fresh-water trout.

As shown in Figure 2, the biological half-life (T ½) of non-bound I-131 in the plasma of trout is 1.7 days. Hickman (1962), found that the T ½ (I-131) for whole blood was 202.6 and 279.4 hours for two fresh-water starry flounder, calculated from data obtained 30 and 70 hours after injection.

It should be noted (Table II) that almost all I-131 in the plasma is in the ionic form even five days post-injection. Although the amount of protein-bound I-131 is low, it is rather significant because it is non-hormonal. Leloup and Fontaine (1960) previously have shown that iodide will bind directly to trout plasma proteins *in vitro*. In the present study, by the fifth day, however, the I-131 in the protein-bound (PBI) fraction increases and this increase is probably due to the appearance of labeled thyroxine. Tong *et al.* (1961) indicate that labeled thyroxine was not detectable in the plasma of hagfish until the fourth day after injection. It is apparent from this and other work (Hickman, 1962) that great care should be taken in using plasma PBI as a parameter of thyroid activity in fish.

Our data, presented in Table III, indicate that per unit time some 3.8 times more I-131 is excreted in the urine than is lost by the gills in fresh-water rainbow trout. Observations by Marshall (see Beiter, 1933) indicated more iodide was excreted by the gill than by the kidney in toadfish. These conflicting results are not entirely unexpected since the trout has a glomerular kidney while that of the toadfish is aglomerular. Also the activity of the gills in osmoregulation is quite different in the two species.

Since the I-131 in the plasma of trout is mostly ionic, the glomerular filtrate should have about the same concentration of I-131 as the plasma. The urine plasma ratios of I-131 indicate that the I-131 of the glomerular filtrate is mostly reabsorbed. Bricker and Hlad (1955), Giebisch *et al.* (1956) and Williamson *et al.* (1962) have shown that in mammals, iodide reabsorption is a passive diffusion process taking place mainly in the proximal tubule. The rainbow trout kidney has a proximal tubule; however, the lack of correlative measurements precludes any conclusion as to the nature of the reabsorptive process in trout.

Another parameter of renal function, I-131 clearance, was determined. In studies of the clearance of any substance by the kidney, the plasma level of that substance should remain reasonably constant throughout the test period. The short test period, and the fact that little I-131 was lost from the fish indicate that this condition was fulfilled in our studies. Our experiments were designed to get information on the maximal (diuretic) and normal values for I-131 clearance by the kidney of a fresh-water salmonid. Diuresis was induced by exposing trout to distilled water, thereby increasing the osmotic gradient across the gills. Tap water was used as a control since its composition approximates that of Michigan trout waters. Published values for urine flow in rainbow trout range from 60–110 ml. kg./day. Holmes and McBean (1963) have found the GFR (glomerular filtration rate) of rainbow trout at 12° C. to be 170 ml./kg./day; thus, some 30%–60% of the glomerular filtrate is excreted per day. Values for urine flow obtained in this study suggest that the trout were diuretic and therefore the measured values can be considered to be near maximal.

The average iodide clearance for trout exposed to distilled water was three times that of trout injected with Na I-131 and then exposed to tap water (Table III). The increased clearance may have been due to differences in uptake (the distilled water animals had accumulated I-131 from distilled water which contained a small amount of Na I-131, whereas the tap water animals received an intraperitoneal injection of Na I-131) or more probably due to the greater diuretic stress imposed on the fish in distilled water.

It is quite apparent that the kidney of fresh-water trout is of major importance for the excretion of iodide. Reabsorption of filtered iodide is relatively efficient, as indicated by low urine/plasma ratio; however, the efficiency is somewhat less than that for chloride. Fromm (1963) has obtained a chloride clearance value of 6.8 ml./kg./day for rainbow trout whereas an iodide clearance of 12.6 ml./kg. day was obtained in the present study.

The authors wish to express their gratitude to Dr. E. P. Reineke and Dr. W. D. Collings for their helpful suggestions.

SUMMARY

1. NaSCN inhibits I-131 uptake by the lower jaw (thyroid) and increases the rate of loss of I-131 from trout, presumably by way of the gills.

2. The biological half-life (T ½) of ionic I-131 in the blood plasma of trout is 1.7 days.

3. I-131 is excreted in the urine and by the gills, with the urinary loss being 3.8 times that of the gill per unit time.

4. The I-131 urine/ plasma ratios indicate that the trout kidney absorbs much of the filtered iodide.

LITERATURE CITED

Berg, O., A. Gorbman and H. Kobayashi, 1959. The thyroid hormones in invertebrates and lower vertebrates. *In:* Comparative Endocrinology (A. Gorbman, Ed.): pp. 302–319.

Bricker, N. S., and C. J. Hlad, Jr., 1955. Observations on the mechanism of the renal clearance of I-131. *J. Clin. Invest.*, **34**: 1057–1072.

Chavin, W., 1956. Thyroid distribution and function in the goldfish, *Carassius auratus*. *J. Exp. Zool.*, **133**: 259–275.

Fromm, P. O., 1963. Studies on renal and extra-renal excretion in a fresh-water teleost. *Comp. Biochem. Physiol.*, **10**: 121–126.

Giebisch, G., M. B. McLeod and F. Kavaler, 1956. Renal excretion of radioiodide in the dog. *Amer. J. Physiol.*, **187**: 529–535.

Hickman, C. P., Jr., 1959. The osmoregulatory role of the thyroid gland in the starry flounder, *Platichthys stellatus*. *Canad. J. Zool.*, **37**: 997–1060.

Hickman, C. P., Jr., 1962. Influence of environment on the metabolism of iodide in fish. *Gen. Comp. Endocrinol., Suppl.*, **1**: 48–62.

Holmes, R. M., 1961. Kidney function in migrating salmonids. *Rep. Challenger Soc. Camb.*, **3**: 23.

Holmes, W. N. and R. L. McBean, 1963. Studies on the glomerular filtration rate of rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.*, **40**: 335–341.

Krogh, A., 1939. Osmotic Regulation in Aquatic Animals. Cambridge University Press.

Leloup, J., 1952. Action des antithyröidiens sur la fixation de l'iode radioactif et la synthèse de la thyroxine dans la thyroide de deux téléostéens marins. *C. R. Soc. Biol.*, **146**: 1017–1020.

LELOUP, J., AND M. FONTAINE, 1960. Iodine metabolism in lower vertebrates. *Ann. N. Y. Acad. Sci.*, **86**: 316–353.

MAQSOOD, M., E. P. REINEKE AND P. O. FROMM, 1961. *In vitro* I-131 uptake by tissues of rainbow trout. *Physiol. Zoöl.*, **34**: 34–38.

MARINE, D., AND C. H. LENHART, 1910a. On the occurrence of goitre (active thyroid hyperplasia) in fish. *Bull. Johns Hopkins Hosp.*, **21**: 95–98.

MARINE, D., AND C. H. LENHART, 1910b. Observations and experiments on the so-called thyroid carcinoma of brook trout (*Salvelinus fontinalis*) and its relation to ordinary goitre. I. *J. Exp. Med.*, **12**: 311–337.

MARSHALL, E. K.; IN BEITER, R. N., 1933. Further studies concerning the action of diuretics upon the aglomerular kidney. *J. Pharmacol. Exper. Therap.*, **49**: 250–256.

PICKFORD, G. E., AND J. W. ATZ, 1957. The Physiology of the Pituitary Gland of Fishes. N. Y. Zoological Soc., New York, N. Y.

ROBERTSON, O. H., AND A. L. CHANEY, 1953. Thyroid hyperplasia and tissue iodine content in spawning rainbow trout: A comparative study of Lake Michigan and California sea-run trout. *Physiol. Zoöl.*, **26**: 328–340.

TONG, W., P. KERKOF AND I. L. CHAIKOFF, 1961. I-131 utilization by thyroid tissue of hagfish. *Biochem. Biophys. Acta*, **52**: 299–304.

WILLIAMSON, H. E., J. R. SCRANTON, F. N. MARSHALL AND N. S. HALMI, 1962. Stop flow analysis of renal tubular site of reabsorption of radioiodide in dogs. *Proc. Soc. Exp. Biol. Med.*, **109**: 23–26.

ACTIVE TRANSPORT OF D-GLUCOSE BY INTESTINAL SEGMENTS, IN VITRO, OF ICTALURUS NEBULOSUS[1, 2]

X. J. MUSACCHIA, S. S. NEFF AND D. D. WESTHOFF

Department of Biology, Saint Louis University, St. Louis 4, Missouri, and the Marine Biological Laboratory, Woods Hole, Massachusetts

In vitro preparations of mammalian intestine have been found useful in studies of absorption and active transport. Thorough reviews of this subject have been published recently (Crane, 1960; Wilson, 1962). It is clearly evident that, in comparison to mammals, interest in other vertebrates has been minimal. There are only a few reports of studies of absorption utilizing *in vitro* intestinal preparations made from fish.

Wilson (1957), using *in vitro* intestinal sac preparations, reported active transport of proline and glycine in the intestine of the puffer, *Spheroides maculatus,* and a lack of transport of glucose in similar intestinal preparations from sea robin, *Prionotus carolinus,* scup, *Stenotomus chrysops,* toad fish, *Opsanus tau,* and the puffer, *Spheroides maculatus.* Carlisky and Huang (1962) studied the intestinal mucosa of the dogfish, *Squalus acanthias.* They used an *in vitro* preparation of mucosal sheets, and concluded that mechanisms for the active transport of glucose were operative.

In a series of pilot experiments using intestinal sac preparations made from both fresh-water and marine species, Musacchia and Fisher (1960) reported active transport of D-glucose in catfish, *Ictalurus nebulosus* (formerly *Ameiurus nebulosus*), and perch, *Perca flavescens.*

The objectives of the current study were to extend the use of *in vitro* intestinal sac preparations in investigations of intestinal absorption in fish, and to describe some features of absorption, particularly characteristics of active transport in the intestine of the bullhead catfish, *Ictalurus nebulosus.*

MATERIALS AND METHODS

Collection and maintenance of catfish

Catfish were collected during summer months from local ponds (*e.g.,* Ice House Pond) in Woods Hole, Massachusetts. Eel pot traps were baited with squid or quahogs and were inspected almost daily. Catches varied from none to about a dozen. Average body size was about 6 to 8 inches; however, they ranged from 4 to 12 inches. Specimens were transferred to the laboratory and into

---

[1] This work was supported by O.N.R. Research Contract No. 1490, administered by the Marine Biological Laboratory, Woods Hole, and Saint Louis University Cancer Research Institutional Committee, CRIC 16 and 33. We are grateful to Dr. T. Hastings Wilson, Department of Physiology, Harvard Medical School for his comments and suggestions concerning the manuscript.

[2] Presented before the American Physiology Society, April, 1963 (Fed. Proc. 22: 227).

fresh-water aquaria at 22–24° C. These aquaria were continuously aerated and flushed with running water. The fish were not fed and they were maintained in this manner for one or two days before experimentation.

Intestinal sac and chemical methods

The intestinal sac preparation was comparable to that described by Crane and Wilson (1958) and modifications have been described elsewhere (Musacchia and Westhoff, 1964). Immediately after spinalectomy, the abdominal cavity was opened and intestine from the pylorus to the rectum was surgically removed, freed of serosal connective tissue and everted.

At all times the intestine was bathed and handled in chilled fresh-water teleost Ringer's solution, made according to the formulae of Forster and Taggert (1958) and now routinely prepared and supplied by the Chemical Department of the Marine Biological Laboratory.

Everted intestine was used whole (W) or divided into two or three segments of about 5 cm. each, depending on the size of each fish. Intestinal segments were designated as upper (U), middle (M), or lower (L), depending on anatomical location. Since the "small" and "large" intestine in fish are for the most part indistinguishable, we refer to these preparations as intestinal segments and do not designate them as rigidly as with mammalian preparations. The "upper" segment was always the portion adjacent to the stomach and would be considered comparable to the duodenum of mammals. The "lower" segment represented the terminus of the gut but ordinarily did not include that portion identifiable as the rectum or lower bowel (Barrington, 1957). The "middle" segment was merely a portion taken from between the upper and lower areas.

The intestinal sac preparation was made by tying one end of the everted gut to a glass cannula and ligating the opposite end. Thus, a sac was made fast to the glass cannula, filled with 1 ml. of solution (on the serosal side) and suspended in a graduated centrifuge tube with 8 ml. of solution (on the mucosal side). In routine experiments the initial concentration of D-glucose on each side of the intestinal wall was the same. Graduated centrifuge tubes were used as incubating chambers for the intestinal sacs. These were used to determine changes in fluid volume at the termination of each run. Under the experimental conditions employed, there were no detectable alterations in fluid volume on either side of the intestinal wall.

Concentrations of D-glucose, 5 or 10 mg. /100 ml., were made up in the appropriate fresh-water teleost Ringer's solution. When phlorizin (5×10^{-4} M) was used, it was added to the Ringer's-glucose media prior to experimentation. A concentration of 5×10^{-4} M phlorizin was selected because of its demonstrated capacity to interfere with absorption of D-glucose in preparations made from a variety of mammals (Wilson, 1962), and an elasmobranch (Carlisky and Huang, 1962).

Ordinarily each preparation was continuously oxygenated with 95% oxygen, 5% carbon dioxide, and incubated at 22–24° C. In each case a five-minute equilibration period was allowed prior to a 20-, 30-, or 40-minute run. The incubation temperatures of 22–24° C. were selected since these are representative of the temperatures at which these fish were caught and maintained.

During experiments involving anaerobiosis, 95% oxygen, 5% carbon dioxide, was replaced with 100% nitrogen. The solutions were continuously gassed with nitrogen before and during the entire run.

In the course of the low-temperature experiments, catfish were acclimated for about four days in a cold room at 0–4° C. Intestine was removed and prepared in the same cold room and incubated for a period of 20 minutes at 0–2° C., when the first aliquot was taken from each side. The same preparations, after a five-minute period for equilibration, were again incubated at 22–24° C. for an additional 20 minutes, and then second aliquots were taken.

D-glucose concentrations in the mucosal and serosal compartments were analyzed colorimetrically by standard methods, using a glucose oxidase reaction (Glucostat from Worthington Biochemical Corporation). Colorimetric methods were used also for analyses of D-xylose (Brown, 1946) and D-fructose (Roe *et al.*, 1949). Wet and dry weights of the tissue were determined by analytical weighing and drying in an oven at 105° C. for at least 24 hours.

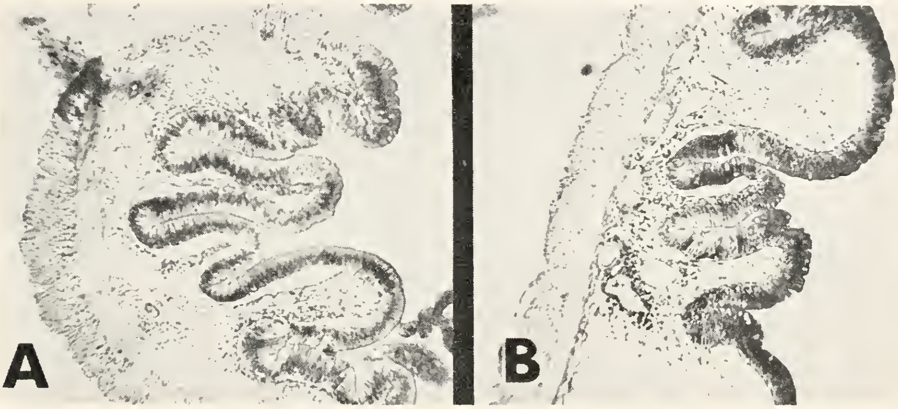


FIGURE 1. Cross-section of everted intestine from *Ictalurus nebulosus* before incubation, A, and B, after 30 minutes' incubation with teleost Ringer's solution and 10 mg./100 ml. D-glucose, at 22–24° C. Each 100 ×.

Histological sections were made routinely; tissue samples were taken before and after experimental runs, fixed in Bouin's solution, sectioned and stained with hematoxylin and eosin.

Under the experimental conditions, the tissue retained its integrity (Fig. 1). The photomicrographs showed that a 30-minute exposure in the incubation medium does not seriously alter the intestinal wall. The epithelium remains intact although there was some distortion of the submucosa in a number of the preparations.

Despite the fact that many of the fish were infected with intestinal parasitic worms (they were recorded on our data sheets), there did not appear to be any influence or relation to the functional characteristics measured. The only feature of note is that sometimes a leak or break in the intestinal wall occurred during an experimental run, and these runs were discarded.

RESULTS

In two series of experiments conducted in 1961, with starting concentrations of 5 or 10 mg. 100 ml. D-glucose on each side of the intestine, there was uptake from the mucosal side and an increase in concentration on the serosal side (Table 1). One of the criteria for active transport was therefore evident, *i.e.*, movement of the sugar against an apparent concentration gradient. Blank preparations

TABLE 1

Active transport of D-glucose in intestine, in vitro, *of the catfish* Ictalurus nebulosus; *where movement of sugar is against a concentration gradient, there is a fall in concentration on the mucosal side and a rise on the serosal side. Changes on each side are shown as (−) = decrease or (+) = increase. Mean and S.E.M. are given. Effects of anaerobiosis, 100% nitrogen, and phlorizin, 5 × 10^{-4} M, are shown*

Year	Experimental preparations*	μMoles D-glucose gm. tissue (dry wt.) 30 min.	
		Mucosal side	Serosal side
1961	5 mg. % D-glucose		
	Upper (6)	9.4 ± 2.5 (−)	10.4 ± 2.6 (+)
	Middle (6)	20.5 ± 2.7 (−)	27.7 ± 7.9 (+)
	Lower (6)	19.2 ± 7.1 (−)	12.3 ± 4.2 (+)
	10 mg. % D-glucose		
	Upper (9)	43.8 ± 4.3 (−)	19.8 ± 3.4 (+)
	Middle (8)	71.3 ± 12.6 (−)	44.8 ± 10.1 (+)
	Lower (6)	19.0 ± 3.2 (−)	16.9 ± 8.1 (+)
1962	Control 10 mg. % D-glucose		
	Upper (7)	21.3 ± 2.3 (−)	11.0 ± 2.1 (+)
	Lower (7)	28.6 ± 5.2 (−)	7.4 ± 4.2 (+)
	Nitrogen 10 mg. % D-glucose		
	Upper (8) Group A	44.3 ± 6.7 (−)	7.1 ± 2.3 (+)
	Lower (5)	41.8 ± 6.2 (−)	6.4 ± 2.0 (+)
	Upper (4) Group B	27.4 ± 8.6 (−)	4.7 ± 1.7 (−)
	Lower (5)	14.6 ± 5.2 (−)	12.5 ± 6.3 (−)
	Nitrogen 0% D-glucose**		
	Upper (4)	5.0 ± 1.5 (+)	2.7 ± 0.7 (+)
	Lower (5)	4.5 ± 0.7 (+)	2.6 ± 1.3 (+)
	Phlorizin I*** 10 mg. % D-glucose		
	Upper (8)	21.9 ± 5.5 (+)	5.8 ± 1.2 (+)
	Lower (8)	14.4 ± 3.8 (−)	14.7 ± 5.1 (+)
	Phlorizin II*** 10 mg. % D-glucose		
	Upper (7)	37.5 ± 10.7 (+)	13.5 ± 9.0 (+)
	Lower (7)	23.5 ± 4.4 (−)	21.9 ± 3.2 (+)

* Number of segments in parentheses.

** Blank runs.

*** Phlorizin I on mucosal side only, Phlorizin II on mucosal and serosal side. Only upper segments were treated with phlorizin.

were run intermittently, *i.e.*, intestinal sacs in teleost Ringer's solution without D-glucose, and after 30 and 40 minutes in only a few instances was there more than a trace of the sugar in the serosal fluid. Thus, corrections to the values recorded (Table I) were not considered essential.

In order to further test the possibility that mechanisms for active transport were operative, a group of three experiments with varying concentrations on both sides of the intestine was also carried out. In one series 10 mg./100 ml. D-glucose was placed only on the mucosal side and a blank solution (*i.e.*, only teleost Ringer's solution) on the serosal side. In these preparations, there was mucosal uptake and serosal increase. In another series, the mucosal side had the blank solution and 10 mg./100 ml. D-glucose was placed on the serosal side. In these preparations, there was neither serosal increase nor did any D-glucose appear in the mucosal medium. A series was also run wherein a lesser D-glucose concentration, 5 mg./100 ml., was placed in the mucosal medium and a 10 mg./100 ml. solution was placed in the serosal medium. Even with this two-fold concentration difference in the two intestinal compartments, there was mucosal uptake and serosal increase in D-glucose concentration. This last group of three experiments was summarized in Figure 2.

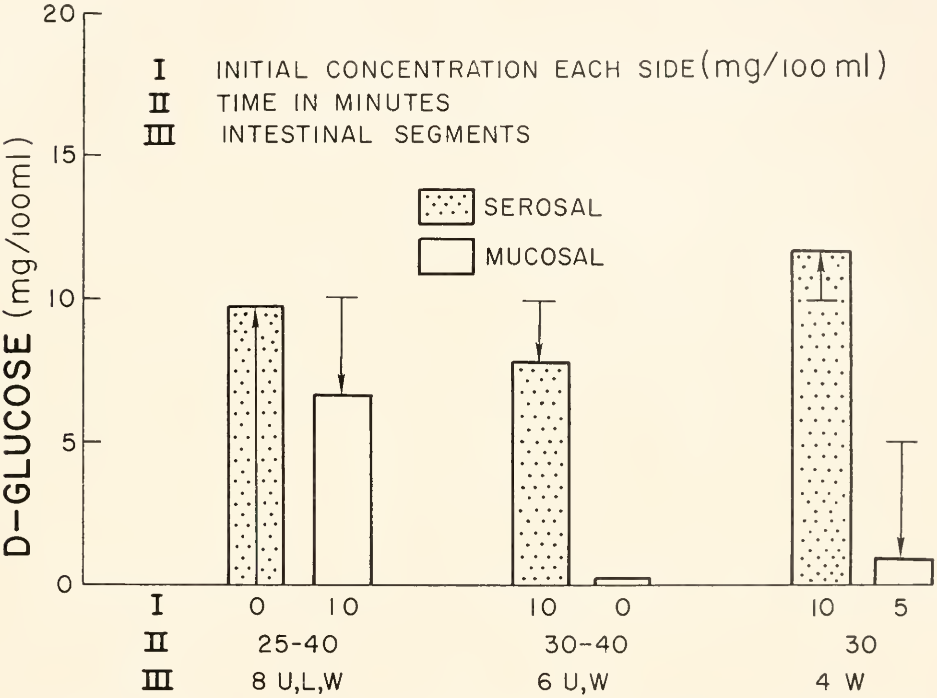


FIGURE 2. Results with various initial concentrations of D-glucose on each side of the intestinal wall. Intestinal sacs *in vitro*, 22–24° C., from catfish, *I. nebulosus*. Arrows indicate starting concentration and direction of change.

TABLE II

Effects of low temperature on active transport of D-glucose in intestine, in vitro*, *of the catfish* Ictalurus nebulosus. *Changes are shown as (−) = decrease and (+) = increase. Mean and S.E.M. are given*

Starting conc. Time and temp. Incubation	μMoles D-glucose gm. tissue (dry wt.) 30 min.		Terminal S/M ratio**
	Mucosal side	Serosal side	
10 mg. % D-glucose			
1st 20 min. 0–2° C.	9.0 ± 1.7 (−)	3.7 ± 1.7 (+)	1.2 ± 0.1
2nd 20 min. 22–24° C.	37.4 ± 1.4 (−)	7.3 ± 1.3 (+)	2.8 ± 0.3

* 13 whole (W) specimens were used.

** S/M ratio is terminal concentration of D-glucose in serosal medium/terminal concentration in mucosal medium.

Catfish intestinal preparations often showed considerable tolerance of anoxic conditions. In an experiment with 10 mg./100 ml. D-glucose, inhibition of the unidirectional change could be shown in only 9 (Group B) out of 22 intestinal segments tested (Table I). In 13 segments (Group A), even under 100% nitrogen, there was a notable level of D-glucose uptake from the mucosal side, and increased amounts of the sugar on the serosal side. In blank preparations, *i.e.*, no D-glucose in the incubation medium, after the usual 30-minute run under nitrogen gassing, some glucose appeared in both compartments.

TABLE III

Catfish intestinal sacs, in vitro, *with D-xylose*

Starting conc. Time and temp. Incubation	Animal no. and segment*	Final values in mg. 100 ml.	
		Mucosal side	Serosal side
10 mg. %			
40 min.	X1 U	8.50	8.56
27° C.	L	8.41	8.44
	X2 U**	9.37	1.00
	L**	9.72	1.12
10 mg. %			
30 min.	X3 W	9.89	10.54
23.5° C.	X4 W	9.38	10.18
	X5 W	8.95	9.20
20 mg. %			
30 min.	X6 W	20.30	20.74
23.5° C.	X7 W	21.09	20.30
30 min.	X8 U**	19.05	2.05
27° C.	L**	19.46	1.11

* Intestinal segment: U = upper, L = lower, W = whole.

** D-xylose only on mucosal side, serosal side "blank."

Phlorizin

Phlorizin, 5×10^{-4} *M*, was found to inhibit absorption of D-glucose, 10 mg./100 ml., in preparations of intestine from catfish (Table I). Use of various experimental approaches, *i.e.*, phlorizin only on the mucosal side (Phlorizin I), or in both mucosal and serosal compartments (Phlorizin II), served to confirm inhibitory effects. In both types of phlorizin-treated catfish intestinal preparations, D-glucose was given off to each side. This was particularly evident when phlorizin was placed on both the mucosal and the serosal side (Phlorizin II). The use of paired segments for comparisons, wherein upper segments were treated with phlorizin and lower segments from the same animal were control runs, served further to validate the inhibitory effects of the glycoside.

Temperature effect

When intestinal preparations were incubated at 0–2° C., there was inhibition of both uptake and transport of D-glucose (Table II). The same segments at

TABLE IV

Catfish intestinal sacs, in vitro, *with D-fructose. Levels of glucose also measured*

Starting conc. Time and temp. Incubation	Animal no. and segment*	Final values in mg./100 ml.			
		Fructose		Glucose	
		Mucosal side	Serosal side	Mucosal side	Serosal side
30 mg. %					
30 min.	F1 W	30.87	29.40	0.33	2.74
21° C.	F2 W	29.40	27.76	0.00	0.56
12° C. (A)**	F3 W	29.40	28.14	0.00	1.62
30 min.					
30° C.	F4 W	30.69	26.96	0.35	1.76
12° C. (A)**	F5 W	30.14	26.96	0.26	0.88
45 min.	F6 W	30.33	25.95	0.00	0.40
24.5° C.	F7 W	30.55	26.87	0.07	1.87
50 mg. %					
30 min.					
24° C.	F8 W	47.40	43.37	0.25	2.24
12° C. (A)**	F9 W	48.70	46.75	0.19	0.97
30 min.	F10 W	(lost)	41.80	(lost)	0.13
24.4° C.	F11 W	50.60	45.61	0.00	0.47
45 min.					
30° C.	F12 W	48.96	44.48	0.26	1.76
12° C. (A)**	F13 W	48.96	46.55	0.44	0.61

* Intestinal segment: W = whole.

** Temperature of aquaria (A) 4 to 5 days, (ordinarily, 22–24° C.).

22–24° C. showed a four-fold increase in uptake of D-glucose from the mucosal medium and a two-fold increase in concentration in the serosal medium (Table II).

D-xylose

There was no active transport of D-xylose (Table III), when 10 or 20 mg./100 ml. D-xylose was placed on both sides of the intestinal wall. During incubation periods of 40 minutes there was some tendency, on both surfaces, to take up from 1 mg.% to 1.5 mg.% D-xylose (Table III). However, after 30 minutes, concentrations were practically unchanged. The extremely low level of uptake of this sugar was further evidenced in the two pairs of experiments, (X2 U** and L**, and X8 U** and L**) where D-xylose 10 or 20 mg./100 ml. was placed only on the mucosal side. After periods of 30 or 40 minutes slightly more than traces appeared in the serosal compartment.

D-fructose

There was no evidence of active transport of fructose, despite the various combinations of incubation temperatures and incubation periods (Table IV). There was a somewhat consistent trend of more serosal uptake of the sugar, as compared with slight or no uptake of fructose on the mucosal side. The concomitant analysis for D-glucose in both serosal and mucosal media showed a range from non-detectable to 2.7 mg.%. In many cases where 1 mg.% or 2 mg.% D-glucose was found on the serosal side, there was only a slightly greater uptake of D-fructose (Table IV).

DISCUSSION

Under the conditions described, D-glucose is absorbed from the mucosal compartment and increased in the serosal compartment, a unidirectional change. These changes support the contention that the sugar is being moved against a concentration gradient, one of the primary characteristics of active transport phenomena in intestinal segments, *in vitro*.

The obvious differences in amounts of D-glucose absorbed and transported in the 1961 and 1962 experiments are of some note. Laboratory conditions and techniques were similar. However, poikilotherm vertebrates are subject to seasonal variations and climatic influences which can modify any number of functional or morphological characteristics. We noted, for example, that during the summer of 1962, about 90% of the catfish collected were smaller than those collected in 1961. Collections were made from the same sites and in the same manner of random trapping. Whether the small size was a reflection of maturity or nutritional restrictions is not known and extended speculation would not explain the differences. Suffice it to say that until additional studies are conducted, to show otherwise, our conclusion is that differences were inherent in these populations and some of these were evident in the results obtained. In mammalian preparations, starting concentrations of glucose are much greater: 450 to 500 mg./100 ml. for rat intestine (Fisher and Parsons, 1949, 1950), 300 mg./100 ml. for hamster intestine (Wilson and Wiseman, 1954) and 100 mg./100 ml. for ground squirrel intestine (Musacchia and Neff, 1963). For catfish intestine, starting concentrations were considerably lower (5 and 10 mg./100 ml.). Possibilities of utilizing higher concentrations, comparable with those used in mammalian preparations, were ruled out in 1960 during our earliest pilot experiments with fish intestine (Musacchia and Fisher, 1960). A series of *in vitro* experiments of intestinal transport of sugars, using another poikilotherm, *Chrysemys picta*, also showed that low concentrations (1 to 18 mg./100 ml.) were most useful (Fox and Musacchia, 1960; Fox, 1961a, 1961b).

In general, values for the S/M ratios (*i.e.*, the ratio of D-glucose terminal concentration in the serosal medium to the terminal concentration in the mucosal medium) for these fish intestinal preparations were lower than those ordinarily obtained in mammalian preparations (Wilson *et al.*, 1960). In the catfish intestine, with starting concentrations of D-glucose 10 mg./100 ml., average S/M ratios were 2.27 (upper) and 1.46 (lower).

It is notable that many segments of catfish intestine showed an ability to continue active transport under apparently anaerobic conditions. Furthermore, average S/M values for control preparations, 2.27 (upper) and 1.46 (lower), and anaerobic preparations, 2.65 (upper) and 1.85 (lower), were essentially similar. Reports of anaerobic conditions, *in vitro*, have shown inhibition of active transport of glucose (Darlington and Quastel, 1953; Wilson and Wiseman, 1954; Wilson, 1962). In each of these investigations mammalian intestinal segments were studied, wherein active transport is generally recognized as being oxygen-dependent. The question herein raised is relevant to this situation in fish. Do fish intestinal tissues require oxygen levels comparable to mammalian preparations, or is it possible that some energy-yielding reactions responsible for active transport are anaerobic (*e.g.*, greater utilization of glycolytic pathways) and sufficient to provide energy for active transport of D-glucose in catfish intestine? It is also possible that under the conditions described minute amounts of residual oxygen may still be available.

As a point of interest, *I. nebulosus* is a bottom-dweller in fresh-water ponds, an environment generally with low oxygen tensions. It is possible that these observed functional characteristics of catfish intestine may be a reflection of overall metabolic adjustment to environmental oxygen limitations.

A concentration of the glycoside, phlorizin, 5×10^{-4} *M*, has been found to be an effective inhibitor of D-glucose active transport by intestinal preparations (Newey, Parsons and Smyth, 1959; Carlisky and Huang, 1962). It is of some note that in the catfish preparations the mode of action, although herein unidentified, was apparently uniform, *viz.*, sugar absorption was inhibited, and intestinal tissue reacted by leaking out considerable amounts of endogenous D-glucose. Thus, catfish intestine reacts to phlorizin in a manner comparable with intestinal preparations made from other animals.

Under the influence of low temperatures there is evident inhibition of processes involved in movement of sugar through the intestinal wall. The evident release from inhibition can be seen when in the same segments, during a subsequent period of incubation at the usual temperatures of 22–24° C., there is a resumption of the undirectional change, *i.e.*, uptake from the mucosal medium and increased concentrations in the serosal medium. This shows, in addition, that exposure to low temperatures does not alter the viability of the tissue so as to abolish absorption.

Fox (1961a) also found that low temperatures inhibit the rate of transport in intestinal sacs from the turtle, *Chrysemys picta*. By way of comparison, when intestinal segments were made from cold torpid turtle and incubated with D-glucose (4.5 mg./100 ml.) at 2° C., glucose from the tissue increased the concentrations in the mucosal media. Catfish preconditioned for a few days at 0–4° C. can be likened to turtles in cold torpor. In these fish, despite the low temperatures, there does not appear to be any tissue "release" of D-glucose. This suggests that concentrations of free sugar may be minimal in catfish intestinal tissue or that absorption mechanisms involved in the unidirectional change, herein postulated, may be sufficiently operative, even at low temperature, to overshadow the appearance of limited amounts of endogenous D-glucose.

The results with D-xylose support the view that this sugar does not undergo active transport in the intestine. Thus, in this regard catfish intestine is comparable with turtle (Fox, 1961a) and hamster (Wilson and Vincent, 1955) intestine.

Fructose is generally accepted as being in an intermediary position between the rapidly absorbed sugars, *e.g.*, glucose and galactose, and the slowly absorbed sugar, xylose.

Wilson and Vincent (1955) reported that in sacs of hamster intestine there was no movement of fructose against a concentration gradient. The results obtained with catfish intestine were comparable with those obtained by Wilson and Vincent (1955) using hamster intestine, and Fox (1961a) using turtle intestinal preparations. In general, in catfish preparations, fructose concentrations fell on both sides of the intestinal wall and, in some preparations, glucose was found in both compartments. The greater concentrations of glucose, however, often appeared in the serosal compartment. It is interesting that Fox (1961a), using *in vitro* preparations of turtle intestine with D-fructose in various concentrations on both sides of the wall, also reports some appearance of glucose in the serosal medium and suggests the possibility of fructose conversion to glucose. We suggest that fructose active transport is non-existent in catfish intestine, particularly under the conditions herein described, and, furthermore, conversion to glucose is minimal. In keeping with the view that two major end-products, glucose and lactic acid, reflect utilization of fructose, another promising experiment might lie in longer periods of incubation and examination of lactic acid production.

In comparative physiology the need for recognition of a functional characteristic in a wide variety of animals is as important as the quantitation of that activity in the same animal. Thus, one more species, the catfish, *I. nebulosus*, may be added to the growing list of animals in which active transport of sugar in the intestine has been demonstrated.

SUMMARY

1. Active transport of D-glucose was shown in intestinal sac *in vitro* preparations made from the bullhead catfish, *Ictalurus nebulosus*. D-xylose and D-fructose did not undergo active transport.

2. Population and/or seasonal differences in absorption characteristics were evident in studies from year to year.

3. Low temperatures, 0–2° C., or phlorizin, 5×10^{-4} M, inhibit the absorption of D-glucose.

4. Nitrogen (100%) anaerobiosis was inhibitory to D-glucose, 10 mg./100 ml., active transport in less than half of the intestinal segments tested, and with 5 mg./100 ml. there was no inhibition of active transport.

5. In an extension of tissues which can be credited with exhibiting active transport, catfish intestine may now be cited in comparative physiology.

LITERATURE CITED

Barrington, E. J. W., 1957. The alimentary canal and digestion. *In:* Brown, M. E. (ed.); The Physiology of Fishes, Volume 1—Metabolism. Academic Press, Inc., New York.

Brown, A., 1946. Determination of pentose in the presence of large quantities of glucose. *Arch. Biochem.*, **11**: 269–278.

Carlisky, N. J., and K. C. Huang, 1962. Glucose transport by intestinal mucosa of the dogfish. *Proc. Soc. Exp. Biol. Med.*, **109**: 405–408.

Crane, R. K., 1960. Intestinal absorption of sugars. *Physiol. Rev.*, **40**: 789–825.

Crane, R. K., and T. H. Wilson, 1958. *In vitro* method for the study of the rate of intestinal absorption of sugars. *J. Appl. Physiol.*, **12**: 145–146.

Darlington, W. A., and J. H. Quastel, 1953. Absorption of sugars from isolated surviving intestine. *Arch. Biochem. Biophys.*, **43**: 194–207.

Fisher, R. B., and D. S. Parsons, 1949. A preparation of surviving rat small intestine for the study of absorption. *J. Physiol.*, **110**: 36–46.

Fisher, R. B., and D. S. Parsons, 1950. Glucose absorption from surviving rat small intestine. *J. Physiol.*, **110**: 281–293.

Forster, R. P., and J. V. Taggert, 1958. Use of isolated renal tubules for the examination of metabolic processes associated with active cellular transport. *J. Cell. Comp. Physiol.*, **36**: 251–270.

Fox, A. M., 1961a. Transport of monosaccharides by intestinal segments of the painted turtle, *Chrysemys picta*. *Comp. Biochem. Physiol.*, **3**: 285–296.

Fox, A. M., 1961b. Active transport of C^{14}-D-glucose by turtle small intestine. *Amer. J. Physiol.*, **201**: 295–297.

Fox, A. M., and X. J. Musacchia, 1960. *In vitro* studies of intestinal absorption in the turtle, *Chrysemys picta*. *Fed. Proc.*, **19**: 182.

Musacchia, X. J., 1960. Some characteristics of active transport of sugars by intestinal segments of *Ameiurus nebulosus*. *Biol. Bull.*, **119**: 328.

Musacchia, X. J., and S. S. Fisher, 1960. Comparative aspects of active transport of D-glucose by *in vitro* preparations of fish intestine. *Biol. Bull.*, **119**: 327–328.

Musacchia, X. J., and S. S. Neff, 1963. Active absorption of D-glucose by intestinal segments of the ground squirrel, *Citellus tridecemlineatus*. *Comp. Biochem. Physiol.*, **9**: 37–40.

Musacchia, X. J., and D. D. Westhoff, 1964. Absorption of D-glucose by segments from active, hibernating, irradiated and non-irradiated ground squirrels, *Citellus tridecemlineatus*. *Finn. Acad. Sci.* (in press)

Newey, H., B. J. Parsons and D. H. Smyth, 1959. The site of action of phlorrhizin in inhibiting intestinal absorption of glucose. *J. Physiol.*, **148**: 83–92.

Roe, J. H., J. H. Epstein and N. P. Goldstein, 1949. A photometric method for the determination of inulin in plasma and urine. *J. Biol. Chem.*, **178**: 839–845.

Wilson, T. H., 1957. *In vitro* studies on intestinal absorption of fish. *Biol. Bull.*, **113**: 362.

Wilson, T. H., 1962. Intestinal Absorption. Pp. 263. W. B. Saunders Company, Philadelphia.

Wilson, T. H., and G. Wiseman, 1954. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *J. Physiol.*, **123**: 116–125.

Wilson, T. H., and T. N. Vincent, 1955. Absorption of sugars *in vitro* by the intestine of the golden hamster. *J. Biol. Chem.*, **216**: 851–866.

Wilson, T. H., E. C. C. Lin, B. R. Landau and C. R. Jorgensen, 1960. Intestinal transport of sugars and amino acids. *Fed. Proc.*, **19**: 870–875.

THE ACCUMULATION OF NITROGENOUS COMPOUNDS IN TERRESTRIAL AND AQUATIC EGGS OF PROSOBRANCH SNAILS

WILLIAM C. SLOAN

Department of Biology, San Diego State College, San Diego 15, California

Pomacea paludosa (Say) and *Marisa cornuarietis* (Linn.) are fresh-water snails of the family Ampullariidae. These genera have many common morphological and ecological features (*cf.* Michelson, 1955, for a discussion of the genera of this family) but differ strikingly in that eggs of *Marisa* are aquatic while those of *Pomacea* are terrestrial. Shelled, calcareous eggs of *Pomacea* are deposited on emergent vegetation and, in the laboratory, embryos develop and hatch without contact with water. The eggs of *Marisa*, which are shell-less and imbedded in a gelatinous matrix, are deposited on submerged vegetation.

The importance of embryonic habitat in determining patterns of nitrogen excretion has been stressed by Needham (1929, 1931). The present study makes use of the relatively controlled, yet natural, situation described above in which two related species differ markedly only with respect to embryonic habitat. This provides an opportunity for a more critical evaluation than is usually possible of the role of environment in determining nitrogenous excretory patterns.

Different developmental stages of eggs were analyzed for uric acid, ammonia and urea. Results are consistent with the generally accepted view (*cf.* Prosser and Brown, 1961 for extensive documentation) that nitrogen excretion is a labile character readily modifiable by environmental demands. A report on nitrogenous excretory products of the adult snails will follow.

METHODS

Eggs of *Pomacea* were collected in the field and kept in the laboratory until desired stages of development were reached. After being examined to confirm developmental stage and viability, groups of 25–50 eggs were dropped into phosphate buffer composed of equal parts of 0.1 *M* alkaline and acid phosphate, pH 6.8 (Conway, 1958). Eggs were then homogenized for three minutes with a motor-driven Teflon pestle. Homogenates were made to volume with buffer, deproteinized after the procedure followed by Gregg and Ballentine (1946) and centrifuged at 1500 *g* for ten minutes. Aliquots of the supernatant were taken for ammonia and urea analyses. Uric acid analysis required extraction in glycine buffer at pH 9.4 (Dubbs *et al.*, 1956). Analyses for this compound therefore were made on separate groups of eggs. These were selected as described above, homogenized and brought to volume with buffer. In order to obtain clear preparations for the spectrophotometric analysis to follow, relatively high centrifugal forces (20,000 *g* for ten minutes) were applied to the homogenates. Aliquots of supernatant were taken for analysis.

TABLE I

Recovery from standard solutions. Values in micrograms of nitrogen

Compound	Analytical method	Analyses	Present	Found
Ammonium sulfate	Diffusion	19	0.64	0.64 ± 0.01*
Urea	Diffusion following urease action	29	0.57	0.56 ± 0.04

* Standard deviation.

Marisa eggs were deposited in the laboratory and kept in aerated spring water until needed. Fine-tipped glass pipettes were inserted into capsules and fluid removed by suction. Small embryos were sucked up along with fluid but older ones were removed with jeweler's forceps. Capsular contents were homogenized in appropriate buffers and further preparations carried out as described for eggs of *Pomacea.*

Ammonia analyses were carried out according to the diffusion method of Shaw and Beadle (1949). Urea was determined by treating samples with a commercial urease preparation (Nutritional Biochemicals Corporation) in phosphate buffer. Treated samples were incubated for one hour at 25° C., after which alkali was added and diffusion accomplished as for ammonia. Uric acid was assayed spectrophotometrically according to the method of Kalckar (1947), in which the compound is destroyed by its specific enzyme, uricase.

Tables I and II indicate the degree of reliability of the analytical methods used for ammonia, urea and uric acid in the ranges under consideration.

TABLE II

Recovery of uric acid added to whole egg homogenates. Values in micrograms per ml

Initial	Added	Expected	Found
2.3	16.0	18.3	18.1
2.0	16.0	18.0	17.8

RESULTS

Table III shows results of analyses performed on eggs in two stages of development. Stage I represents eggs within the first three days of development, while Stage II refers to those within one or two days of hatching. Eggs of *Marisa* hatched within nine to twelve days under laboratory conditions, while those of *Pomacea* usually required a day or so longer.

As the table indicates, increase in ammonia content of *Marisa* eggs is significant while uric acid remains the same. The small values sometimes recorded for urea were inconsistent and only rarely exceeded controls. These values were judged, on the basis of familiarity with the analytical technique used, to be due to variability of the method in this low range. It was therefore considered that these values did not lend themselves to statistical comparison and such was not performed in this case. The possibility of loss of ammonia from developing eggs was investigated. Since an initial attempt to culture *Marisa* eggs to hatching

TABLE III

Accumulation of nitrogenous end-products by eggs. Values are means followed by standard deviations and represent micrograms of nitrogen found per ten eggs

		Stage I	Stage II	*P*
Marisa	Ammonia-N	0.10 ± 0.08 (6*; 28†)	0.48 ± 0.13 (6; 20)	<0.005
	Urea-N	0.01 ± 0.06 (6; 28)	0.12 ± 0.30 (6; 20)	—
	Uric acid-N	0.88 ± 0.63 (4; 18)	0.70 ± 0.36 (3; 25)	>0.50
Pomacea	Ammonia-N	1.20 ± 0.76 (9; 46)	3.23 ± 0.21 (4; 34)	<0.005
	Urea-N	0.25 ± 0.30 (9; 46)	0.00 (3; 34)	—
	Uric acid-N	2.9 ± 1.7 (6; 25)	13.5 ± 5.2 (5; 33)	<0.025

* Number of analyses performed.

† Mean number of eggs per analysis.

in an antibiotic solution was unsatisfactory, short-term observations (to minimize bacterial production or utilization of ammonia) without antibiotics were made. Groups consisting of two-, six- and ten-day-old eggs were placed in separate small flasks with spring water. As eggs could not be freed completely of their gelatinous covering without injury, some of this material was present with the eggs in each experimental flask. In order to compensate for this, a comparable amount of egg-free gelatinous coat was added to control flasks. All flasks were aerated with ammonia-free air and after 24 hours aliquots from each were taken for analysis. No loss was detected from the younger eggs but a small loss from Stage II eggs, amounting to 0.14 μg. ammonia-N per ten eggs per day, was discovered. It is quite possible that additional loss occurs over the entire period of development but that the amount lost from early stages in a 24-hour period is below the limit of detection.

While content of both ammonia and uric acid increases significantly during development of *Pomacea* eggs, five times more uric acid nitrogen accumulates than ammonia nitrogen. Values for urea were again quite small and variable and are not treated statistically. In order to account for possible loss of volatile ammonia from eggs of this species, newly laid eggs were placed in dry, tightly stoppered bottles through which an ammonia-free air stream was led into flasks containing standard acid. After 14 days, at which time hatching began, acid from control and experimental flasks was titrated. No difference was found and therefore volatile ammonia is not lost from the egg in quantities detectable by the present method. As the surface of the egg is normally dry, it does not seem likely that non-volatile loss occurs.

DISCUSSION

Although *Pomacea* eggs accumulate approximately the same amount of uric acid relative to dry weight of eggs (7.1 mg.% of dry weight) as do those of *Limnaea stagnalis* (4.0 mg.%; Baldwin, 1935), the uricotelic status of the latter species is in doubt (Needham, 1935; Spitzer, 1937). Extent of accumulation in *Limnaea* cannot, therefore, be used as a criterion by which the question of uricotelism in *Pomacea* can be judged. *Helix* is generally considered to be uricotelic in the adult stage (Bricteux-Gregoire and Florkin, 1962). The per cent by weight of uric acid reported to be accumulated by embryos of *H. aspersa* (0.28%; Needham, 1935) is far in excess of that recorded for *Limnaea* and *Pomacea*. However, the incubation period for *Helix* is about twice that of *Pomacea;* the amount accumulated by *Helix* over incubation periods comparable to those of *Pomacea* and *Limnaea* is not known. Terrestrial eggs of another ampullariid (*Pila virens*) have been studied by Meenakshi (1955), who reported that uric acid does accumulate in these eggs. This compound was estimated only qualitatively, however, and further comparisons cannot be made.

As the above summary indicates, comparative quantitative information from other gastropod species is insufficient to weigh heavily in the interpretation of data presented here for *Pomacea* and *Marisa*. Because eggs of *Pomacea* accumulate approximately five times as much uric acid nitrogen as ammonia nitrogen during development and do not store detectable quantities of urea, embryos are believed to be uricotelic. Developing eggs of *Marisa* accumulate neither urea nor uric acid. They do, however, accumulate significant quantities of ammonia, as well as lose this substance to the medium. These observations lead to the conclusion that embryos of *Marisa* are ammonotelic.

This paper formed part of a doctoral thesis submitted to the Graduate School of the University of Florida. I am indebted to Dr. Robert M. DeWitt, under whose guidance the study was carried out. My thanks go to Dr. Myra Keen of Stanford University for determination of current taxonomic status of the snails investigated.

SUMMARY

1. Observations on the relative amounts of nitrogenous compounds accumulating in developing eggs lead to the conclusions that embryos of *Marisa cornuarictis* are ammonotelic, those of *Pomacea paludosa* uricotelic.

2. These results, obtained on related snails differing conspicuously only in embryonic habitat, emphasize the highly adaptive nature of nitrogen-excretion patterns.

LITERATURE CITED

BALDWIN, E., 1935. The energy sources in ontogenesis. VIII. The respiratory quotient of developing gastropod eggs. *J. Exp. Biol.*, **12**: 27–35.

BRICTEUX-GREGOIRE, S., AND M. FLORKIN, 1962. Les excreta azotés de l'escargot *Helix pomatia* et leur origine metabolique. *Arch. Int. Physiol. Biochem.*, **70**: 496–506.

CONWAY, E. J., 1958. Microdiffusion Analysis and Volumetric Error. Macmillan Publishing Co.

DUBBS, C., F. DAVIS AND W. ADAMS, 1956. Simple microdetermination of uric acid. *J. Biol. Chem.*, **218**: 497–504.

GREGG, J. R., AND R. BALLENTINE, 1946. Nitrogen metabolism of *Rana pipiens* during embryonic development. *J. Exp. Zool.*, **103**: 143–168.

KALCKAR, H., 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. I. Determination of hydroxypurine compounds. *J. Biol. Chem.*, **167**: 429–443.

MEENAKSHI, V., 1955. Protein catabolism in the developing egg of *Pila virens*. *Current Sci.*, **24**: 199–200.

MICHELSON, E. H., 1955. Studies on the biology of the genus *Ceratodes* (Mollusca, Pilidae). (Dissertation, Harvard University).

NEEHAM, J., 1929. Protein metabolism and organic evolution. *Science Progress,* **23**: 633–648.

NEEDHAM, J., 1931. Chemical Embryology. Volume II. Cambridge Univ. Press.

NEEDHAM, J., 1935. Problems of nitrogen catabolism in invertebrates. II. Correlation between uricotelic metabolism and habitat in the phylum Mollusca. *Biochem. J.,* **29**: 238–251.

PROSSER, C. L., AND F. A. BROWN, JR., 1961. Comparative Animal Physiology. Second Ed. W. B. Saunders Co., Phila.

SHAW, J., AND L. C. BEADLE, 1949. A simplified ultra-micro Kjeldahl method for the estimation of protein and total nitrogen in fluid samples of less than 1.0 μl. *J. Exp. Biol.,* **26**: 15–23.

SPITZER, J., 1937. Physiologisch-ökologische Untersuchungen über den Exkretstoffwechsel der Mollusken. *Zool. Jahrb. Abt. Allg. Zool. Physiol.,* **57**: 457–496.

THE REGULATION OF LIVER TRYPTOPHAN PYRROLASE ACTIVITY DURING THE DEVELOPMENT OF RANA CATESBIANA[1]

MELVIN SPIEGEL AND EVELYN SCLUFER SPIEGEL

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire, and the Marine Biological Laboratory, Woods Hole, Massachusetts

Tryptophan pyrrolase (peroxidase-oxidase), the enzyme which catalyzes the formation of formylkynurenine from tryptophan, has been found in the livers of a variety of mammals but not in other tissues (Knox, 1955). Knox and Mehler (1950) have demonstrated that the activity of the enzyme was greatly increased after the injection of tryptophan. Increased liver enzyme levels could, however, be produced by a variety of conditions other than injection of substrate. Such substances as histidine and tyrosine (Knox and Mehler, 1951) and histamine and adrenalin (Knox, 1951) when injected into intact animals produced increased activity of this enzyme. In adrenalectomized animals, liver enzyme activity was increased by injections of adrenocorticotropic hormone (Geschwind and Li, 1953) and the glucocorticoid hormones (Thompson and Mikuta, 1954; Knox and Auerbach, 1955). Knox and Auerbach (1955) concluded that there were probably two types of inducing agents, the substrate and an adrenal hormone.

More recently, Feigelson and Greengard (1961a) have demonstrated that rat liver tryptophan pyrrolase is activated by an iron protoporphyrin, probably hematin, located in the microsomes. Greengard and Feigelson (1961) further demonstrated that, following substrate induction, the enzyme was more fully activated or saturated by its hematin prosthetic group but following cortisone induction, this increased activation did not take place. These results further indicated alternative mechanisms for the initiation of enzyme induction by substrate and the steroid hormones. This conclusion has been substantiated by the recent work of Greengard, Smith and Acs (1963). These authors demonstrated that both inductions were prevented by puromycin, an inhibitor of protein synthesis. Actinomycin D, however, an inhibitor of RNA synthesis, prevented the hydrocortisone induction of tryptophan pyrrolase but did not prevent the induction of this enzyme by its substrate.

Nemeth and Nachmias (1958) and Auerbach and Waisman (1959) have demonstrated that mammalian tryptophan pyrrolase is present only in the adult liver and is absent in fetal liver. Nemeth (1959) measured enzyme activity and its enhancement by tryptophan injection in the liver of the guinea pig, rabbit, and rat in fetal and postnatal stages, including the adult. Injections of tryptophan did not increase enzyme activity in fetal liver, and the response to injection in all species developed simultaneously with the rapid increase of enzyme activity to adult levels. Constitutive enzyme, therefore, had to be present before induction

---

[1] Supported by grant G 21810 from the National Science Foundation.

could take place. Nemeth (1959), as a result of these investigations, concluded that the lack of response of fetal liver to tryptophan injections, as well as to adrenocortical hormones, suggests that substrate induction and adrenocortical secretion are not the mechanisms controlling the increase in enzyme activity during development.

There are two reports in the literature demonstrating substrate induction of tryptophan pyrrolase activity in early development. These are the reports by Stearns and Kostellow (1958) and Kostellow (1961), using dissociated embryonic cells of *Rana pipiens.* These investigators were not able to detect enzyme activity in intact embryos before hatching but enzyme activity was detected in all cells of embryos dissociated into cell cultures at a stage prior to gastrulation and incubated in L-tryptophan. If L-tryptophan was omitted from the culture medium no activity was detected. As gastrulation proceeded, the ability to be induced by L-tryptophan became progressively restricted to the lightest (in terms of density) endoderm cells.

Kato (1959) attempted to repeat this work on *Rana pipiens* embryonic cells using Stearns and Kostellow's procedures, Barth and Barth's procedures for the preparation and cultivation of dissociated embryonic cells, and dejellied, but not dissociated, hemisected pre-gastrulae cultivated in Barth and Barth's medium. Kato demonstrated that tryptophan pyrrolase and formylase activity were *not* induced by substrate in any of the three types of culture.

Furthermore, Stearns and Kostellow (1958) were not able to detect β-galactosidase activity until some time after hatching. They were, however, able to substrate-induce this enzyme in cells obtained from post-gastrula embryos and regarded this observation as premature activity. Kato, on the other hand, was able to find definite β-galactosidase activity in stage 12 embryos (Shumway, 1940) but was not able to induce further activity in dissociated cells incubated in substrate. Spiegel and Frankel (1961, and unpublished results) have obtained results similar to those of Kato. Kato has suggested that Stearns and Kostellow's results may have been due to a contamination of their cultures by bacterial cells which would have seriously affected the outcome of this sort of experiment.

Recently Greengard, Smith and Acs (1963) have reported that the adrenalectomy of young rats shortly after birth does not affect the developmental increase of tryptophan pyrrolase activity. They suggest that this result indicates that a substrate-type rather than an adrenocortical secretion-type of regulation is involved in the development of tryptophan pyrrolase activity. They also demonstrated that treatment of young rats with actinomycin D had no effect on the postpartum rise of tryptophan pyrrolase activity. The role, therefore, of substrate-induction in controlling or regulating the synthesis of tryptophan pyrrolase is still open to question.

The results reported by Spiegel and Frankel (1961) with *Rana pipiens* embryos indicated that tryptophan pyrrolase activity was not detectable through stage 25 (Shumway, 1940) although activity in the amphibian adult liver was comparable to that found in mammalian adult liver, (Spiegel, 1961). It was of interest, therefore, to determine when activity does first appear during amphibian development and to determine, if possible, what the factors are which regulate the enzyme's synthesis and activity. Young tadpoles of *Rana pipiens* are very small and it is necessary in order to assay for tryptophan pyrrolase to either homogenize the entire tadpole or to pool and assay the livers tediously dissected from a large number of tadpoles. The former procedure is open to a number of legitimate criticisms. For example, failure to detect activity may reflect either a dilution of activity by the "excess" tissues, or inhibition of liver enzyme activity by the "excess" tissues. The detection of activity may not be solely due to liver enzyme but to other sources. The latter procedure requires a great deal of time and the livers dissected earlier may lose activity during the accumulation of a sufficient number to do an assay. Freezing of livers would not obviate this difficulty, for Spiegel and Spiegel (unpublished results) have demonstrated a loss of activity in adult amphibian livers following this treatment.

For these reasons, therefore, tadpoles of *Rana catesbiana*, which have considerably larger livers than those of *Rana pipiens*, were chosen for this study. Tryptophan pyrrolase activity was determined through metamorphosis. During the course of this investigation, further confirmation of the results of Kato and of Spiegel was obtained.

Materials and Methods

Larval stages and adults of the frog *Rana catesbiana* were collected in Massachusetts by the Supply Department of the Marine Biological Laboratory from ponds located in the Sippewissett area of Falmouth, Massachusetts. In Hanover, New Hampshire, larval stages were collected by the senior author from Occom Pond. Larvae and adults were maintained in tap water at room temperature. The water was changed three times per week.

For embryonic stages, eggs were obtained by pituitary injection of large adult females and fertilized by the usual method of stripping directly into a suspension of macerated testes in 10% Holtfreter's solution, pH 7.8 (Holtfreter, 1931). The embryos were then washed three times with sterile 10% Holtfreter's solution, pH 7.8, and incubated at 18–22° C. until the desired stage was attained.

Since there is not any published description of developmental stages of *Rana catesbiana,* those of Shumway (1940) for the embryonic stages of *Rana pipiens* and of Taylor and Kollros (1946) for the larval stages of *Rana pipiens* were applied to corresponding stages of *Rana catesbiana.* This procedure has been adopted by Brown and Cohen (1958) and others.

From the time of hatching until the cessation of feeding during metamorphosis, tadpoles were fed an excess of either Gaines Dog Meal (General Foods Corporation, Battle Creek, Michigan) or Purina Rabbit Chow (Ralston Purina Co., St. Louis, Missouri) three times per week.

Through stage 14 (Shumway, 1940), the method of Friedberg and Eakin (1949) of cutting embryos into halves and quarters to permit penetration of substrate was used. Accordingly, the jelly and vitelline membrane were removed by dissection with watchmaker forceps. Intact embryos, halves, and quarters were incubated for 12–24 hours in either full-strength Holtfreter's solution or 0.03 *M* L-tryptophan (in full-strength Holtfreter's solution). Cut embryos remained alive as indicated by normal closure of the cut surface in both media. After five washings with the control medium, tryptophan pyrrolase activity was measured in 12.5% homogenates by the Knox and Auerbach method (1955) as modified slightly by Spiegel (1961). For stage 22 embryos 10% Holtfreter's solution was substituted for the full-strength solution. In larval stages and in adults, livers were dissected out and liver enzyme activity assayed in 12.5% homogenates as described above. It is important to note that, under these conditions, enzyme activity was directly proportional to concentration of frog liver homogenate. The Knox and Auerbach method is based on the conversion of tryptophan to kynurenine which is the product actually measured during the assay. This conversion, however, is a two-step reaction, tryptophan pyrrolase catalyzing the first step, the conversion of tryptophan to formylkynurenine. The enzyme formylase, which catalyzes the conversion of formylkynurenine, the second step, is found in excess in mammalian liver homogenates and is not rate-limiting (Knox and Mehler, 1950). In all control and experimental frog livers and cut or intact embryos, no reaction products absorbing at 321 mμ were detected. This indicated the absence of formylkynurenine, and formylase, therefore, is probably present in excess in frog livers and is not rate-limiting. L-tryptophan, L-histidine (free-base), hydrocortisone acetate, and L-thyroxine were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Hemin was obtained from Mann Research Laboratories, Inc., New York, N. Y. All injections were given intraperitoneally. All enzyme activities were expressed in terms of μmoles of kynurenine formed per hour at 38° C., per gram dry weight of liver (or embryo) ± standard error of the mean.

RESULTS

The results obtained with embryonic stages of *Rana catesbiana* were comparable to those obtained for *Rana pipiens* embryos by Spiegel and Frankel (1961). The data of 9 experiments with 3500 embryos per experiment indicated no constitutive tryptophan pyrrolase activity present in either blastula, mid-gastrula, or early neurula stages, using either intact embryos or halves or quarters of embryos. Furthermore, incubation in L-tryptophan failed to induce enzyme activity in these stages. Measurement of tryptophan pyrrolase activity revealed *no* constitutive enzyme activity in stages 17 and 22 embryos and no indication of activity was noted after tryptophan treatment. In view of the demonstration by Feigelson and Greengard (1961a) that tryptophan pyrrolase is activated by an iron protoporphyrin, probably hematin, the above experiments were repeated with 4 mμmoles of hematin (prepared by dissolving hemin in 0.01 N NaOH) added to the assay mixture. In 5 experiments using 2400 embryos identical results were obtained. There was no constitutive enzyme activity present in the above-mentioned stages and incubation in L-tryptophan failed to induce enzyme activity in these stages.

Table I summarizes the results obtained with larval stages through metamorphosis. It can be seen that enzyme activity in two series of control animals, with and without injection of 0.65% sodium chloride, steadily decreased from approximately 5 μmoles kynurenine per hour per gram dry weight at stage I to essentially zero activity by stage XXIII. In other words, before the onset of metamorphosis and sometime after Shumway stage 22, liver tryptophan pyrrolase activity was roughly that found in the adult liver (Spiegel and Spiegel, unpub-

TABLE I

Liver tryptophan pyrrolase activity of Rana catesbiana *tadpoles*

Developmental stage	Number of animals	Treatment	Activity*
I	92	Tryptophan**	28.08 ± 1.17
	87	0.65% NaCl***	4.45 ± 0.26
	100	No injection	5.53 ± 0.26
XIV	27	Tryptophan	13.32 ± 0.70
	16	0.65% NaCl	4.20 ± 0.17
	8	No injection	3.85 ± 0.39
XVII	8	Tryptophan	8.87 ± 0.29
	8	0.65% NaCl	1.46 ± 0.06
	17	No injection	1.32 ± 0.43
XX	8	Tryptophan	1.16 ± 0.13
	8	0.65% NaCl	1.30 ± 0.25
	15	No injection	0.68 ± 0.12
XXII	8	Tryptophan	1.62 ± 0.31
	8	0.65% NaCl	0.84 ± 0.01
	13	No injection	1.43 ± 0.46
XXIII	16	Tryptophan	2.17 ± 0.04
	11	0.65% NaCl	0.84 ± 0.27
	7	No injection	0.96 ± 0.43
XXV	8	Tryptophan	0.74 ± 0.23
	8	0.65% NaCl	0.98 ± 0.18
	8	No injection	0.94 ± 0.42

* μMoles kynurenine formed at 38° C. per hour per gram of dry weight of liver ± standard error of the mean.

** 1.0 ml. injection of L-tryptophan (110 mg./10 ml. of 0.65% NaCl). Assayed 5 hours later.

*** 1.0 ml. injection. Assayed 5 hours later.

lished results). By the end of metamorphosis, activity in the froglet had virtually disappeared. Table I also demonstrates that substrate-inducibility was correlated with constitutive enzyme activity. Where the constitutive enzyme level was at a high level of activity, *e.g.*, stage I, a 5–6–fold increase in activity was achieved following a single injection of L-tryptophan. At stage XIV, with constitutive enzyme activity at approximately 4 μmoles of kynurenine per hour per gram dry weight, a three-fold increase was noted following L-tryptophan injection. By stage XXV little or no constitutive enzyme activity was detected and no induction was obtained by substrate induction. Experiments with stage XXV embryos, in which 4 mμmoles of hematin were added to the assay mixture as described above, did not produce any detectable activity.

It was thought that perhaps the loss of inducibility during metamorphosis was correlated with the changes in organs of the digestive system (other than the liver) which take place during metamorphosis (Kaywin, 1936). These changes

TABLE II

Effect of varying the time after injection of tryptophan on liver tryptophan pyrrolase activity of tadpoles

Hours after injection*	Stage XIV		Stage XXIII	
	Number of animals	Activity**	Number of animals	Activity
5	10	14.12 ± 0.59	11	1.76 ± 0.13
12	12	14.89 ± 0.43	9	1.59 ± 0.28
17	12	12.54 ± 0.61	15	1.62 ± 0.51
29	13	13.72 ± 0.84	8	1.23 ± 0.09

* 1.0 ml. intraperitoneal injection of L-tryptophan (110 mg./10 ml. of 0.65% NaCl).

** μMoles kynurenine formed at 38° C. per hour per gram dry weight of liver ± standard error of the mean.

could have led to a decrease in the rate of uptake of L-tryptophan following an intraperitoneal injection. The assays described in Table I were carried out 5 hours after injection. Accordingly stages XIV and XXIII tadpoles were injected intraperitoneally with L-tryptophan and their livers assayed 5, 12, 17, and 29 hours after injections. More L-tryptophan should be absorbed as the time after injection increased. It can be noted in Table II, however, that no significant change in enzyme activity was noted during the 29-hour period. These results did not completely rule out the possibility of a change in substrate uptake. It is possible that the substrate was metabolized at such a high rate that, even with increased time for uptake, blood and tissue levels in later stages did not reach levels necessary for induction to take place.

TABLE III

Effect of hydrocortisone and L-histidine on tryptophan pyrrolase activity of tadpole livers

Developmental stage	Number of animals	Treatment	Activity*
XIV	13	Hydrocortisone**	3.42 ± 0.19
	8	Hydrocortisone control***	4.09 ± 0.38
	4	L-Histidine†	5.54 ± 0.26
	4	L-Histidine control††	4.87 ± 0.41
XXIII	4	Hydrocortisone	1.28 ± 0.46
	4	Hydrocortisone control	0.84 ± 0.27
	4	L-Histidine	0.70 ± 0.42
	4	L-Histidine control	0.93 ± 0.56

* μMoles kynurenine formed at 38° C. per hour per gram dry weight of liver ± standard error of the mean. All animals sacrificed and livers assayed 5 hours after intraperitoneal injection.

** 1 ml. injection of hydrocortisone (20 mg./10% ethanol in 0.65% NaCl).

*** 1 ml. injection of 10% ethanol in 0.65% NaCl.

† 1 ml. injection of L-histidine solution prepared by dissolving 38 mg. L-histidine in 5 ml. of 0.65% NaCl + 2 ml. of 0.1 N NaOH, pH adjusted to 7.0 by addition of 0.1 N HCl.

†† 1 ml. injection of 5 ml. of 0.65% NaCl + 2 ml. of 0.1 N NaOH, pH adjusted to 7.0 by addition of 0.1 N HCl.

TABLE IV

Effect of L-thyroxine treatment on tryptophan pyrrolase activity of livers of stage I tadpoles

Treatment	Tadpoles cultured in 3×10^{-8} *M* L-thyroxine for 36 hours		Tadpoles cultured in tap water for 36 hours	
	Number of animals	Activity*	Number of animals	Activity
Tryptophan**	49	18.58	50	36.34
0.65% NaCl***	50	4.18	50	6.89

* μMoles kynurenine formed at 38° C. per hour per gram dry weight of liver.

** 0.1 ml. intraperitoneal injection of L-tryptophan (110 mg./10 ml. 0.65% NaCl) and liver assayed 24 hours later.

*** 0.1 ml. intraperitoneal injection and liver assayed 24 hours later.

In an effort to learn whether this loss of activity during metamorphosis was due to the progressive loss of ability of the adrenal cortex to synthesize steroids, tadpoles were injected with hydrocortisone and L-histidine. These compounds induce tryptophan pyrrolase activity in adult mammalian livers but not in adult *Rana pipiens* livers (Spiegel, 1961). Table III shows that hydrocortisone and L-histidine had no significant effect on the tryptophan pyrrolase activity of stage XIV and XXIII embryos.

Tables IV and V demonstrate that culture of tadpoles in L-thyroxine solutions leads to a 30–50% loss in constitutive enzyme activity within 24–36 hours. They further demonstrate that substrate-inducibility in the tadpole is correlated with constitutive enzyme activity. In animals injected with L-tryptophan and cultured in L-thyroxine, enzyme activity is usually less than half of that in animals injected with substrate and cultured in tap water. The degree of inducibility in

TABLE V

Effect of L-thyroxine treatment on inducibility, by tryptophan, of tryptophan pyrrolase of livers of stage XIV tadpoles*

Number of days cultured in solution	Tadpoles cultured in thyroxine**		Tadpoles cultured in tap water	
	Number of animals	Activity***	Number of animals	Activity
1	4	7.20	2	14.18
2	4	2.63	2	18.39
4	4	8.24	2	17.39
6	4	3.02	2	14.28
8	4	7.79	2	16.69

* Each tadpole was injected intraperitoneally with 1 ml. of L-tryptophan (110 mg./10 ml. 0.65% NaCl) and its liver assayed 5 hours later.

** Tadpoles were cultured in 3×10^{-8} *M* L-thyroxine in tap water (1 tadpole/200 ml. solution) and solution was changed at 24-hour intervals. Controls were treated in same manner with tap water substituted for thyroxine.

*** μMoles kynurenine formed at 38° C. per hour per gram dry weight of liver.

L-thyroxine is considerably reduced and the L-tryptophan injection could not counteract the inhibitory effect of L-thyroxine. This result further supports the repeated observation that the degree of substrate-induction of this enzyme, in the frog, is correlated with the amount of constitutive enzyme in the liver.

In an effort to learn whether the loss of enzyme activity during metamorphosis was due either to a loss of an activator or conversely, to the appearance of an inhibitor, a series of experiments were carried out in which equal volumes of liver homogenates of L-tryptophan-injected stage XIV and XXIII larvae were mixed together. The mixed homogenate, as well as the separate homogenates, were then assayed by the usual procedure. L-tryptophan-injected tadpoles were used to increase the level of activity of the enzyme in stage XIV and, if possible, in stage XXIII so that any marked change in activity of the mixed homogenate would be more reliable and within the sensitivity of the assay method. The results of this experiment are summarized in Table VI. It can be noted that the activity of the stage XIV homogenates was an average of 14.78 μmoles for 10 experiments and that of stage XXIII homogenates 0.71 μmole for 10 experiments. The expected value for the mixed homogenate (the average activity of the two separate homogenates) was 7.75 μmoles. A series of additional experiments with varying proportions of the two homogenates led to the same result; the observed enzyme activity was always less than the expected value. These results suggest the presence of an inhibitor of tryptophan pyrrolase activity in stage XXIII liver homogenates.

TABLE VI

Effect on tryptophan pyrrolase activity of mixing stage XIV and stage XXIII homogenates of livers from L-tryptophan-injected tadpoles*

Type of homogenate**	Number of experiments	Activity***
Stage XIV	10	14.78 ± 0.49
Stage XXIII	10	0.71 ± 0.03
Stage XIV + XXIII****	10	3.25 ± 0.41

* Injected intraperitoneally with 1.0 ml. of L-tryptophan (110 mg./10 ml. 0.65% NaCl) and livers assayed 5 hours later.

** Prepared and assayed by the Knox and Auerbach procedure.

*** μMoles kynurenine formed at 38° C. per hour per gram dry weight of liver ± standard error of the mean.

**** Equal volumes.

DISCUSSION

The observation that liver tryptophan pyrrolase activity appears sometime after stage 22 in *Rana catesbiana* embryos, only to be followed by its virtual disappearance by the end of metamorphosis and reappearance in the adult, is of serious consequence to the developmental biologist who chooses to study the ontogeny of enzymes and structural proteins. Tyler (1957) and Spiegel (1960) have considered the possibility that proteins, during development, may change in their solubility and/or intracellular location. If these events occurred, it would lead to difficult and often misleading interpretations of data of analyses obtained using a single method of extraction. Indeed, Solomon (1959) has elegantly demonstrated changes in solubility for the enzyme, glutamic dehydrogenase, in the developing chick embryo. The finding that an enzyme confined, as far as is known, to a single organ can virtually disappear and then reappear later in development is, as far as the authors are aware, without parallel.

The results obtained in this investigation do not, of course, rule out the possibility that the larval enzyme is a quite different protein(s) from that of the adult as, for example, is the case for fetal and adult hemoglobin (Shelton and Schroeder, 1960) or for the isozymes of lactic dehydrogenase in developing mouse muscle (Markert and Ursprung, 1962). Further investigations of these possibilities are being carried out in our laboratory at the present time.

Regardless of the outcome, the results obtained with tryptophan pyrrolase, the hemoglobins, lactic dehydrogenases, and other proteins amply illustrate the complexity of carrying out studies on the synthesis of proteins in development. In particular, the earlier approach to the problem of ontogenetic changes in proteins, in which organ and tissue extracts containing numerous soluble macromolecules of unknown function are analyzed by a variety of biochemical and immunological techniques, is difficult and perhaps impossible to interpret with meaning. The use of numerous proteins of unknown function(s) for studies in development decreases the probability of interpreting the molecular events involved in the developmental process.

The finding that tryptophan pyrrolase cannot be precociously induced in the bullfrog embryo by its substrate in the absence of demonstrable amounts of constitutive enzyme activity is in agreement with the earlier observation by Spiegel and Frankel (1961) for *Rana pipiens* embryos and by Nemeth (1959) for fetal livers. The demonstration of a positive correlation between constitutive enzyme activity and degree of substrate-inducibility serves as additional confirmation of this observation. It should be made apparent, however, that failure to precociously induce enzyme activity by injection of, or culture in, L-tryptophan does not rule out the possibility of a substrate-type of induction occurring *intracellularly* during development. Secondly, the possibility exists that, under the conditions employed for culture of early embryos, the cells were impermeable to substrate. Finally, it is possible that the failure to induce enzyme activity by substrate in larval stages may be due to a failure of the gut wall and lining of the body cavity to absorb appreciable amounts of L-tryptophan. The fact that tadpoles stop eating during metamorphosis would appear to reinforce this explanation. Enzyme activity would presumably decrease in the absence of food (and presumably of L-tryptophan). An attempt to explore this possibility through the use of perfused livers by the techniques of Goldstein, Stella and Knox (1962) has not been successful thus far. All attempts in our laboratory (unpublished results) of perfusing tadpole and adult frog livers with and without L-tryptophan solutions have led to a rapid loss of constitutive enzyme activity and a failure by substrate to induce or even maintain activity. The demonstration of the presence of an inhibitor in stage XXIII tadpoles indicates that, at least for later stages, a mechanism of inhibition operates rather than the absence of substrate in regulating tryptophan pyrrolase activity.

The failure of either hydrocortisone or of L-histidine to induce liver tryptophan activity in stages XIV and XXIII is not unexpected. Spiegel (1961) has demonstrated that hydrocortisone, L-histidine, cortisone, and adrenocorticotropic hormone (ACTH) fail to induce enzyme activity in the livers of adult *Rana pipiens.* This finding is in direct contrast to the mammalian data in which enzyme activity can be induced in adult livers by these substances. It should be pointed out, however, that Nemeth (1959) failed to obtain induction in mammalian fetal livers (in which constitutive enzyme activity is lacking) by either substrate or adrenocortical hormones. The failure of adrenal steroids to induce activity in the amphibian is indeed puzzling. Schotté and Chamberlain (1955) and Schotté and Bierman (1956) have furnished evidence for the existence of a pituitary-adrenal axis for regenerative processes in the urodele. Regenerative capacities of the hypophysectomized newt were restored when injected with either ACTH or cortisone. Furthermore, Schotté and Wilber (1958) have furnished additional evidence indicating the existence of this synergism in *Rana clamitans* and *Rana pipiens.* Levinsky and Sawyer (1952) have demonstrated that ACTH can partially counteract the decrease in water-balance response to pitocin following hypophysectomy of adult male *Rana pipiens.* The suggestion by Spiegel (1961) that the amphibian adrenal cortical hormones are different from those of the mammal remains as an explanation of the failure of the mammalian hormones to induce enzyme activity. Secondly, the alternative pathway of enzyme induction by steroid hormones, as indicated by the work of Knox and Auerbach (1955) and Greengard, Smith and Acs (1963), may not be present in the amphibian. An analysis of additional steroid-inducible systems in the frog is of importance in this respect.

The observation that a decrease in the constitutive and inducible activity of liver tryptophan pyrrolase occurs following culture of tadpoles in L-thyroxine solution is similar to the decrease in changes in succinoxidase activity of the bullfrog following thyroxine treatment (Paik and Cohen, 1960) and duplicates the naturally occurring event during metamorphosis. While it is attractive to think of thyroxine as the inhibitory agent, little information is available to shed light on this hypothesis. When thyroxine has been added to the assay mixture in varying amounts, no *in vitro* effect has been observed in either larval or adult liver homogenates (Spiegel and Spiegel, unpublished results).

Of extreme importance is the finding that stage XXIII tadpole livers contain an inhibitor of tryptophan pyrrolase activity. The data, however, can not be interpreted in terms of apoprotein concentrations. At least two possibilities exist. Stage XXIII tadpole livers may contain appreciable amounts of apoprotein which is inhibited or, secondly, there may be no significant amounts of apoprotein present. The recent isolation of the apoprotein of mammalian tryptophan pyrrolase by Feigelson and Greengard (1961b), if adaptable to the frog liver, will permit the evaluation of these possibilities. The data do not permit the identification of the inhibitor substance as a repressor of genetic activity (Jacob and Monod, 1961). Their hypothesis is so attractive, however, to the developmental biologist, that further experiments must consider it.

SUMMARY

1. Liver tryptophan pyrrolase activity was studied in embryonic, larval, and adult *Rana catesbiana*. Constitutive enzyme activity appeared after Shumway stage 22, disappeared during metamorphosis, and was again detected in appreciable amounts in the adult.

2. Substrate inducibility was positively correlated with constitutive enzyme activity. In no case was enzyme activity induced by substrate in the absence of detectable constitutive enzyme activity.

3. Culture of tadpoles in thyroxine solutions led to the suppression of enzyme activity.

4. L-histidine and hydrocortisone had no effect on liver tryptophan pyrrolase activity.

5. The appearance, during metamorphosis, of tryptophan pyrrolase activity was described.

6. The implications of these findings were discussed in terms of further experiments on the ontogeny of macromolecules.

LITERATURE CITED

Auerbach, V. H., and H. A. Waisman, 1959. Tryptophan peroxidase-oxidase, histidase, and transaminase activity in the liver of the developing rat. *J. Biol. Chem.*, **234**: 304–306.

Brown, G. W., Jr., and P. P. Cohen, 1958. Biosynthesis of urea in metamorphosing tadpoles. *In:* A Symposium on the Chemical Basis of Development, edited by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., pages 495–513.

Feigelson, P., and O. Greengard, 1961a. A microsomal iron-porphyrin activator of rat liver tryptophan pyrrolase. *J. Biol. Chem.*, **236**: 153–157.

Feigelson, P., and O. Greengard, 1961b. The isolation of the apoprotein of the heme enzyme tryptophan pyrrolase. *Biochim. Biophys. Acta*, **50**: 200–202.

Friedberg, F., and R. M. Eakin, 1949. Studies in protein metabolism of the amphibian embryo. I. Uptake of radioactive glycine. *J. Exp. Zool.*, **110**: 33–46.

Geschwind, I. I., and C. H. Li, 1953. Influence of hypophysectomy and of adrenocorticotropic hormone on a mammalian adaptive enzyme system. *Nature*, **172**: 732–733.

Goldstein, L., E. J. Stella and W. E. Knox, 1962. The effect of hydrocortisone on tyrosine-α-ketoglutarate transaminase and tryptophan pyrrolase activities in the isolated, perfused rat liver. *J. Biol. Chem.*, **237**: 1723–1726.

Greengard, O., and P. Feigelson, 1961. A difference between the modes of action of substrate and hormonal inducers of rat liver tryptophan pyrrolase. *Nature*, **190**: 446–447.

Greengard, O., M. A. Smith and G. Acs, 1963. Relation of cortisone and synthesis of ribonucleic acid to induced and developmental enzyme formation. *J. Biol. Chem.*, **238**: 1548–1551.

Holtfreter, J., 1931. Über die Aufzuch isolierter Teile des Amphibienkeimes. II. Züchtung von Keimen und Keimteilen in Salzlösung. *Arch. f. Entw.*, **124**: 404–466.

Jacob, F., and J. Monod, 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.*, **3**: 318–356.

Kaywin, L., 1936. A cytological study of the digestive system of anuran larvae during accelerated metamorphosis. *Anat. Rec.*, **64**: 413–437.

Kato, Y., 1959. A study of enzyme induction in dissociated embryonic cells of *Rana pipiens* embryos. *In:* Carnegie Institution of Washington Year Book 59, The Lord Baltimore Press, Inc., Baltimore, Md., pages 386–389.

Knox, W. E., 1951. Two mechanisms which increase *in vivo* the liver tryptophan peroxidase activity: specific enzyme adaptation and stimulation of the pituitary adrenal system. *Brit. J. Exp. Pathol.*, **32**: 462–469.

Knox, W. E., 1955. Tryptophan oxidation. *In:* Methods in Enzymology, edited by S. P. Colowick and N. O. Kaplan, Academic Press, Inc., Publishers, New York, N. Y., pages 242–253.

Knox, W. E., and V. H. Auerbach, 1955. The hormonal control of tryptophan peroxidase in the rat. *J. Biol. Chem.*, **214**: 307–313.

Knox, W. E., and A. H. Mehler, 1950. The conversion of tryptophan to kynurenine in liver. I. The coupled tryptophan peroxidase system forming formylkynurenine. *J. Biol. Chem.*, **187**: 419–438.

Knox, W. E., and A. H. Mehler, 1951. The adaptive increase of the tryptophan peroxidase-oxidase system of liver. *Science*, **113**: 237–238.

Kostellow, A. B., 1961. Induced enzyme synthesis in cell cultures of embryonic *R. pipiens*. *Fed. Proc.*, **20**: 222.

Levinsky, N. G., and W. H. Sawyer, 1952. Influence of the adenohypophysis on the frog water-balance response. *Endocrinol.*, **51**: 110–116.

Markert, C. L., and H. Ursprung, 1962. The ontogeny of isozyme patterns of lactate dehydrogenase in the mouse. *Dev. Biol.*, **5**: 363–381.

Nemeth, A. M., 1959. Mechanisms controlling changes in tryptophan peroxidase activity in developing mammalian liver. *J. Biol. Chem.*, **234**: 2921–2924.

Nemeth, A. M., and V. T. Nachmias, 1958. Changes in tryptophan peroxidase activity in developing liver. *Science*, **128**: 1085–1086.

Paik, W. K., and P. P. Cohen, 1960. Biochemical studies on amphibian metamorphosis. I. The effect of thyroxine on protein synthesis in the tadpole. *J. Gen. Physiol.*, **43**: 683–696.

Schotté, O. E., and R. Bierman, 1956. Effects of cortisone and allied adrenal steroids upon regeneration in hypophysectomized *Triturus viridescens*. *Rev. Suisse Zool.*, **63**: 353–375.

Schotté, O. E., and J. Chamberlain, 1955. Effect of ACTH upon limb regeneration in normal and in hypophysectomized *Triturus viridescens*. *Rev. Suisse Zool.*, **62**: 253–279.

Schotté, O. E., and J. F. Wilber, 1958. Effects of adrenal transplants upon forelimb regeneration in normal and in hypophysectomized adult frogs. *J. Embryol. Exp. Morphol.*, **1**: 24–42.

Shelton, J. R., and W. A. Schroeder, 1960. Further N-terminal sequences in human hemoglobins A, S, and F by Edman's phenylthiohydantoin method. *J. Amer. Chem. Soc.*, **82**: 3342–3345.

Shumway, W., 1940. Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.*, **78**: 139–147.

Solomon, J. B., 1959. Changes in the distribution of glutamic, lactic and malic dehydrogenases in liver cell fractions during the development of the chick embryo. *Dev. Biol.*, **1**: 182–198.

Spiegel, M., 1960. Protein changes in development. *Biol. Bull.*, **118**: 451–462.

Spiegel, M., 1961. Tryptophan pyrrolase in the liver of adult *Rana pipiens*. *Biol. Bull.*, **121**: 547–553.

Spiegel, M., and D. L. Frankel, 1961. Role of enzyme induction in embryonic development. *Science*, **133**: 275.

Stearns, R. N., and A. B. Kostellow, 1958. Enzyme induction in dissociated embryonic cells. *In:* A Symposium on the Chemical Basis of Development, edited by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., pages 448–453.

Taylor, A. C., and J. J. Kollros, 1946. Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.*, **94**: 7–23.

Thompson, J. F., and E. T. Mikuta, 1954. Effect of total body X-irradiation on the tryptophan peroxidase activity of rat liver. *Proc. Soc. Exp. Biol. Med.*, **85**: 29–32.

Tyler, A., 1957. Immunological studies of early development. *In:* The Beginnings of Embryonic Development. Amer. Assoc. Adv. of Sci., Washington, D. C., pages 341–382.

THE EFFECT OF THE PATHOGENIC RHIZOPOD HYDRAMOEBA HYDROXENA (ENTZ) ON REPRODUCTION IN CHLOROHYDRA VIRIDISSIMA UNDER VARIOUS LEVELS OF TEMPERATURE[1]

ALAN E. STIVEN

Department of Zoology, University of North Carolina, Chapel Hill, N. C.

Since the discovery by Entz (1912) of *Hydramoeba hydroxena*, which attacks the fresh water polyp hydra, very few attempts have been made to elucidate the ecological and pathological relationships between these organisms; in fact, only those of Reynolds and Looper (1928), Ito (1949, 1950) and Rice (1960) are noteworthy. The ease of culturing large numbers both of host and parasite and the rapidity with which the hydramoebae attack and complete the destruction of many host species suggested a convenient and relatively simple system that would be ideal for studying ecological phenomena associated with population host-parasitism, and for exploring certain epidemiological concepts through controlled experiments. This paper, therefore, is one of a series (Stiven, 1962a; in press) in which this goal has been pursued.

In the analysis of the initiation, progress, and outcome of artificially induced epidemics carried out over several periods of budding in the host population, it is important to know what inhibitory effect the infection has on the host's ability to reproduce, and hence on the reproductive potential and subsequent density of the host population. The relationship between host density and parasite density, and the rate of increase of an epidemic was considered mathematically as early as 1923 by Lotka and later experimentally and mathematically by many others (*e. g.*, Burnett, 1949; Watt, 1959). The consequences of host density and the rate of spread of the infection have also been examined in the hydra-hydramoeba system (Stiven, in press). In this case, however, all buds which detached from parent hydras were immediately removed, thus maintaining the host populations at constant densities throughout any one epidemic. The effect of a changing density of host population through accumulation of buds, and the influence of various key environmental factors on such changing densities have yet to be tested on the rate of spread of the hydramoeba epidemic.

In addition, little is known about the importance of hydramoebae in natural populations of hydra. Hydras apparently undergo seasonal fluctuations in abundance, although relevant studies are rather limited in number (*e.g.*, Welch and Loomis, 1924; Miller, 1936; Bryden, 1952). Miller (1936) reported the presence of the parasite in late summer and early autumn in declining populations of hydras in Douglas Lake, Michigan. Some populations were not noticeably parasitized, whereas others were heavily infected. He concluded that *Hydramoeba* might be an important factor in reducing certain populations, but that it is probably unimportant in determining the quantitative seasonal distribution of hydras. Working in Kirkpatricks Lake in Tennessee, Bryden (1952) briefly mentioned that during the summer of 1949 large numbers of hydramoebae appeared in one of his populations of hydras and that within a short time the entire population disappeared, apparently because of the detrimental effects of the parasite.

---

[1] This study was supported by a National Science Foundation Grant and by a grant from the Research Council of the University of North Carolina.

It was the purpose of this study, therefore, to determine the degree to which different levels (magnitudes) of initial infection with *Hydramoeba* affect the rate of asexual reproduction in *Chlorohydra viridissima* under a range of temperatures compatible with that occurring in their natural environment.

I want to express my sincere appreciation to Dr. C. D. Beers of the Department of Zoology for his helpful comments on this manuscript.

MATERIALS AND METHODS

The common green hydra, *Chlorohydra viridissima*, was the principal host in these experiments, although *Hydra pseudoligactis* was used in one experiment. For experimental use, actively budding hydras were maintained in stock cultures at 21° to 23° C. The experimental treatments comprised four levels of temperature, 15°, 20°, 25°, and 30° C., and three levels of initial infection, a light infection of two hydramoebae per hydra, a heavy infection of 8, and a control of uninfected hosts. There were five individual hydras (replicates) for each combination of temperature and initial infection—a total of 60 hydras. Each host, bearing one completely formed bud, was infected and placed in one of the depressions of a 9-depression spot plate; each depression contained approximately 2 cc. of culture medium. The spot plates were kept in sealed moist chambers throughout the experiments. Each individual hydra was fed *Artemia* (brine shrimp) daily as long as it was able to feed. Egestion products were removed four to five hours after feeding, and the culture medium was changed every 24 hours. The medium was a modified tap-water solution developed by Loomis (1953). Constant illumination was maintained with two 15-watt fluorescent lamps placed two feet from the culture dishes. Preliminary experiments indicated that the responses of the host-parasite system of concern to this study occurred within 10 days; consequently, production of buds by each individual hydra was observed for this period starting from the day of initial infection.

The specimens of *Hydra pseudoligactis* were similarly infected, but only one temperature level (25° C.) was used.

RESULTS

Reduction of budding rate in infected C. viridissima

The average number of buds per hydra per day produced by the five replicates under all treatments during the 10 days of the experimental period is depicted in Figure 1. For example, at 20° C. and in the first day of the experiment, the control group produced an average of 1.0 bud per day per hydra, the lightly infected group 0.8, and the heavily infected group 0.6. By the end of the fifth day the accumulated number of buds per hydra per five days was 4.0, 2.4, 2.0 for the control, light, and heavy infection groups, respectively. By the end of the tenth day these differences had diverged further (8.6, 4.2, and 2.0 buds per

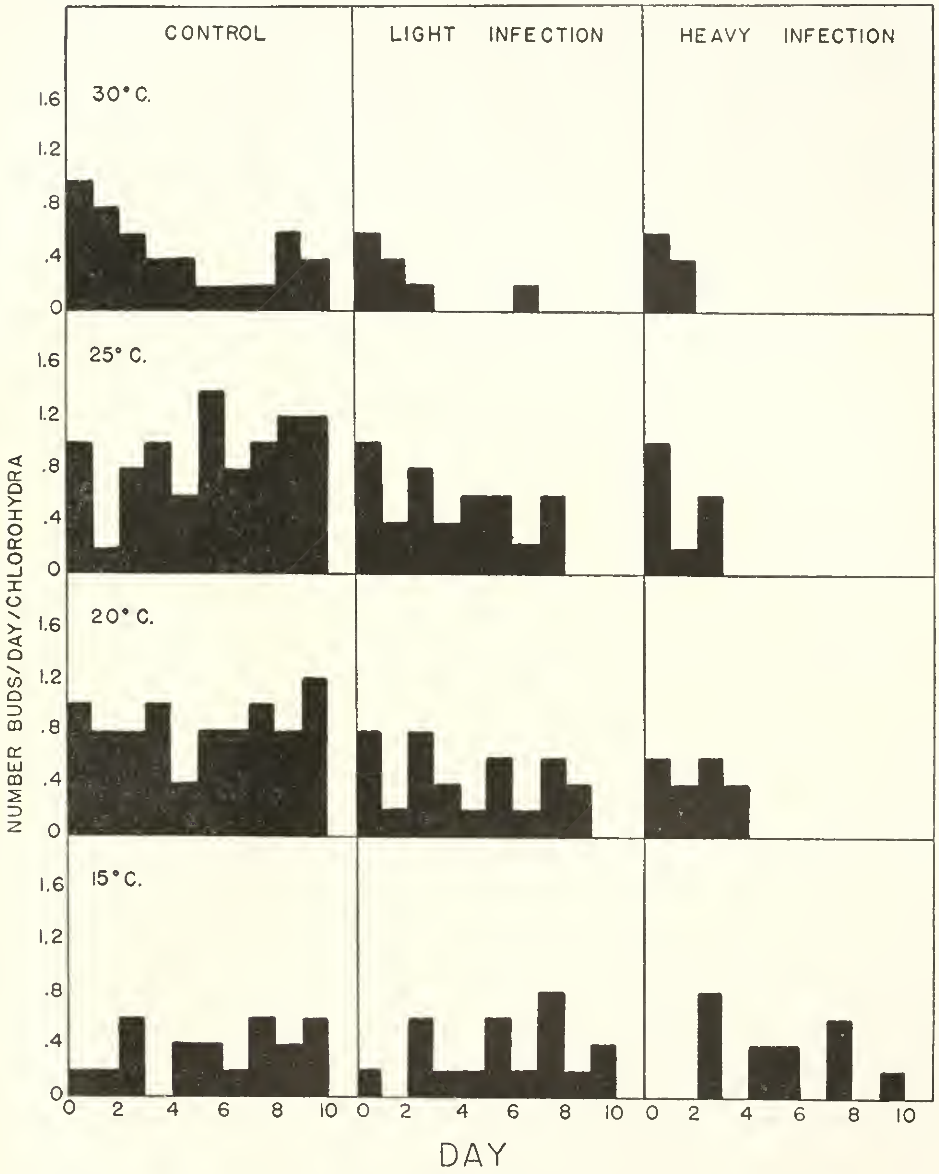


FIGURE 1. Production of buds by infected *Chlorohydra viridissima* during the 10 days of the experiment. Budding rate is represented by the number of buds produced per hydra per day under 4 levels of temperature and 3 levels of initial infection. Each treatment combination consisted of 5 hydras.

hydra per 10 days), since the control group continued to produce buds throughout the entire experiment and the lightly infected group produced them for 9 days.

When the production of buds is compared among the levels of temperature—for example, for the lightly infected group—a sharp drop in bud production occurred at 30° within the first three days of the experiment. At 25° and 20° production of buds also declined, but not as rapidly. At 15°, however, buds were produced throughout the experimental period, but at a lower rate than at 20° and 25° C. This comparison can be expressed quantitatively by summing the daily production of buds for the five replicates of the lightly infected group over the experimental period for the four temperature levels. Thus, the total number of buds per five hydras per 10 days at the respective temperatures (30° through 15°) was 7, 23, 21, and 17. A similar picture is evident for the heavily infected group. At 30°, bud production ceased by the end of the second day. At 25° and 20°, the production of buds ceased at the end of the third and fourth days, respectively. At 15°, however, the infected hydras continued asexual reproduction throughout the 10-day period.

TABLE I

Analysis of variance of total bud production by 5 C. viridissima *during the first two days of the experiment under 4 levels of temperature and 3 levels of initial infection. (4 × 3 factorial design)*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Treatments	11	18.184		
A (Temperature)	3	13.384	4.461	31.5**
B (Infection)	2	3.434	1.717	12.1**
AB Interaction	6	1.366	0.228	1.6 ns
Error	48	6.800	0.142	
Total	59	24.984		

** Significant $P = 0.01$.
ns Not significant at $P = 0.05$.

It is evident that the differences in bud production among the levels of infection and temperature become exaggerated the longer the experiment is carried out, since the controls continue to produce buds, whereas budding in the infected groups either declines or eventually ceases (Fig. 1). The question of importance, therefore, is the significance of the degree of difference in bud production among temperature and infection levels over increasing units of time.

To answer this question, the accumulated number of buds produced during the first two days of the experiment for each replicate under each treatment combination was considered as a simple 4 × 3 factorial experiment. The analysis of variance of these data gives the significance of the effects of temperature and infection, acting alone and in combination (first-order interaction). The results of this analysis are presented in Table I. The significant main effect, temperature, means that the differences in bud production among the various levels of temperature are statistically significant ($P < 0.05$) when averaged over all levels of initial infection. Similarly, the different levels of initial infection have given rise to significant differences in bud production during the first two cumulative days of the experiment when averaged over all levels of temperature. The interaction between temperatures and infection, which is not significant, indicates that these two factors are independent; that is, any simple effect of one factor on budding is not dependent upon the level of the other.

It should be noted that no host mortality occurred in any treatment combination during the first two days of this experiment, and that the differences in budding rate, therefore, are due only to the effects of the hydramoebae.

To determine how these two factors continue to affect the production of buds during the remaining cumulative time-intervals of the experiment, similar analyses of variance were carried out on total bud production by each host for each subsequent cumulative two-day period; that is, from the start of the experiment to the end of the fourth day, from the start to the end of the sixth day, and so on, to the end of the tenth day. This procedure involved four more analyses. The changes in the magnitude of the differences in the effects of the two factors acting separately and in combination are represented by the values of F and are given in Figure 2. Both temperature and infection continued to produce significant effects on the production of buds when examined as single factors. In fact, the degree of significance for infection increases rapidly over time. This was probably due to the early cessation of budding in the heavily infected group. While the interaction was not significant during the first two days

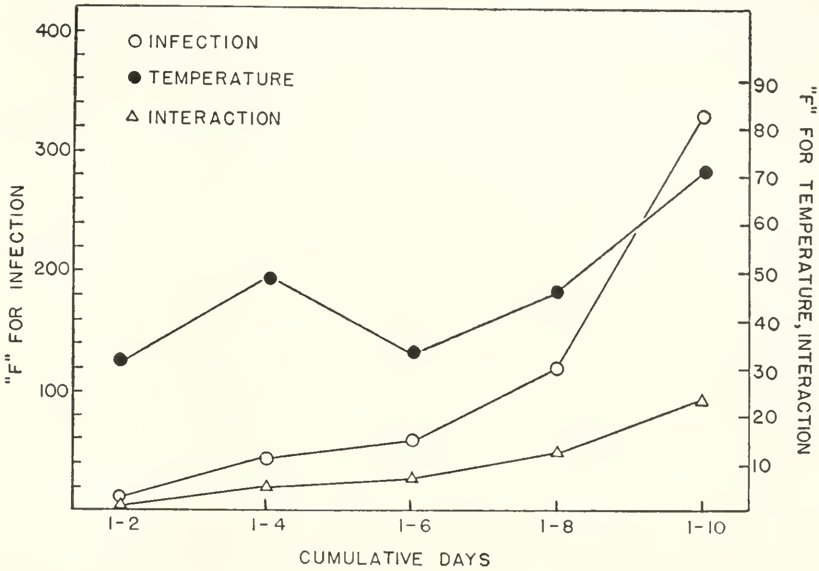


FIGURE 2. Change in the value of F from the analyses of variance of temperature and initial infection effects on bud production. An analysis was performed for each cumulative successive two-day period up to the total of 10 days. The only value of F which is not statistically significant ($P = 0.05$) is the interaction between temperature and infection during the cumulative first two days of the experiment.

of the experiment, significance appeared in the time-interval of one to four days, and gradually increases up to the tenth day. After the second day, therefore, dependence appeared between these two factors, since the difference in bud production among the levels of temperature differed among the three levels of infection beyond day 2. The converse is also true, since an interaction is symmetrical.

If the production of buds is examined only between the light and the heavy infection levels under the four levels of temperature for the cumulative 10-day period (the control group is omitted from the calculations), these two factors and their interaction continue to affect significantly the production of buds ($P < 0.01$), but with a reduction in the value of F in all cases. A clearer picture of this can be derived by examining the contribution of the control group to the variation attributable to temperature and infection. This is done by analyzing the sums of squares and is presented in Table II. When the control is included, over half (62%) of the total variation is caused by initial infection, but when the control is omitted, temperature and infection each contribute approximately one-third to the total variation. In other words, approximately a 25% decrease in the variation attributable to initial infection, and a 17% increase attributable to temperature have occurred. While the control group does influence greatly the significance of the two factors, its removal from the analysis does not alter the significance of temperature and initial infection within the 95% probability bounds.

TABLE II

Proportion of variance attributable to initial infection, temperature, and their interaction when control is included, and excluded, from the analysis of variance. Data for the accumulated 10-day period

Source of variation	Control included		Control excluded	
	Sum of squares	Proportion of variation	Sum of squares	Proportion of variation
A (Temperature)	82.19	0.1990	26.60	0.3715
B (Infection)	255.24	0.6180	25.60	0.3575
AB Interaction	57.16	0.1384	9.00	0.1258
Error	18.40	0.0446	10.40	0.1452
Total	412.99	1.0000	71.60	1.0000

In the preceding analyses the interpretation of the changes in budding rate, represented by the changes in the value of F, is complicated by the fact that after the first two days of the experiment host mortality begins to occur in some of the treatments. This is particularly true at the heavy infection level and at the higher temperature (see Figure 1, and Table III, last column). Obviously, the death of one or several hosts in a particular treatment combination will contribute sizeably to the reduction of the budding rate in that treatment. It becomes very difficult to analyze the complete factorial experiment using only those hosts which have survived to the end of the experiment, since in some treatments all hosts succumbed (see Table III), and the factorial design is destroyed. However, it is possible to analyze the differences in bud production between the light and heavy infection levels for each temperature separately, using only the cumulative time period in which no hosts succumbed. For example, at 15° C. where all hosts survived the entire 10 days, a significant difference ($P < 0.05$) exists between the two infection levels in the mean number of buds per hydra per 10 days. The same result exists at 20° for the first six-day period (hosts started dying after the sixth day in the heavy infection level). On the other hand, at 25° and 30°, no difference exists between the levels of infection in the buds produced per hydra per four days and per two days, respectively. It must be concluded, therefore that the differences in bud production among the levels of infection and temperature are due solely to the inhibitory effect of the hydramoebae only up to the tenth, sixth, fourth, and second days for 15° through 30°, respectively. Beyond these days the reduction in budding is the result of the combination of host mortality and the hydramoebae, with host mortality playing an increasingly important role as temperature increases up to 30°. Host mortality, of course, is due directly to the action of the hydramoebae.

Table III

Certain features of the host-parasite relationship which resulted from the initial infection and contributed to the decline and cessation of budding. Values are the mean ± the standard error. The values in brackets indicate the number of hydras participating in the calculation of the means

Treatment		Day host stopped feeding	Days host host did not feed	Day last bud produced	Day of death of host
Light infection	15°	—	—	—	—
	20°	7.6 ± 1.07 (5)	1.3 ± 0.58 (3)	8.4 ± 0.24 (5)	10.0 ± 0.00 (2)
	25°	6.6 ± 0.68 (5)	1.8 ± 0.48 (4)	7.2 ± 0.58 (5)	10.0 ± 0.00 (1)
	30°	3.0 ± 0.00 (5)	3.0 ± 0.00 (1)	1.6 ± 0.40 (5)	5.5 ± 0.29 (4)
Heavy infection	15°	—	1.0 ± 0.95 (5)	—	—
	20°	5.0 ± 1.10 (5)	4.0 ± 1.41 (2)	3.4 ± 0.22 (5)	8.0 ± 1.00 (3)
	25°	4.2 ± 0.20 (5)	—	2.4 ± 0.40 (5)	5.8 ± 0.20 (5)
	30°	2.2 ± 0.20 (5)	—	1.4 ± 0.24 (5)	3.6 ± 0.40 (5)

Reduction of budding rate in infected Hydra pseudoligactis

As already indicated, observation of budding rate in infected individuals of this species was made only at one temperature level, 25° C. The results are graphed in Figure 3. This species, which has a lower level of resistance to hydramoebae than *C. viridissima* (Stiven, 1962a), ceases budding shortly after infection. Comparing the responses of both species at 25° C. we find almost identical numbers of buds produced by the control groups (47 and 49 buds per 5 hydras per 10 days for *C. viridissima* and *H. pseudoligactis*, respectively). It was shown in an earlier study (Stiven, 1962b) that the former species has a slightly higher ($P < 0.05$) intrinsic rate of increase than *H. pseudoligactis* at 25° C. In fact, at 25° and under daily feeding, a green hydra after 10 days detachment from the parent produces an average of 1.61 buds per day, compared with a value of 1.36 for the brown species. In this experiment, under a light initial infection totals of 22 and 16 buds were produced by the five green and five brown individuals, respectively. Only one green hydra died under this treatment, but all *H. pseudoligactis* individuals succumbed (the average day of death was 6.6). Under a heavy initial infection, identical numbers of buds were produced by both species and the average day of death was 6.2 and 5.4 for the green and brown hydras, respectively. It appears, therefore, that under a heavy initial infection the inherent differences in the level of resistance to the hydramoebae of these two host species are masked.

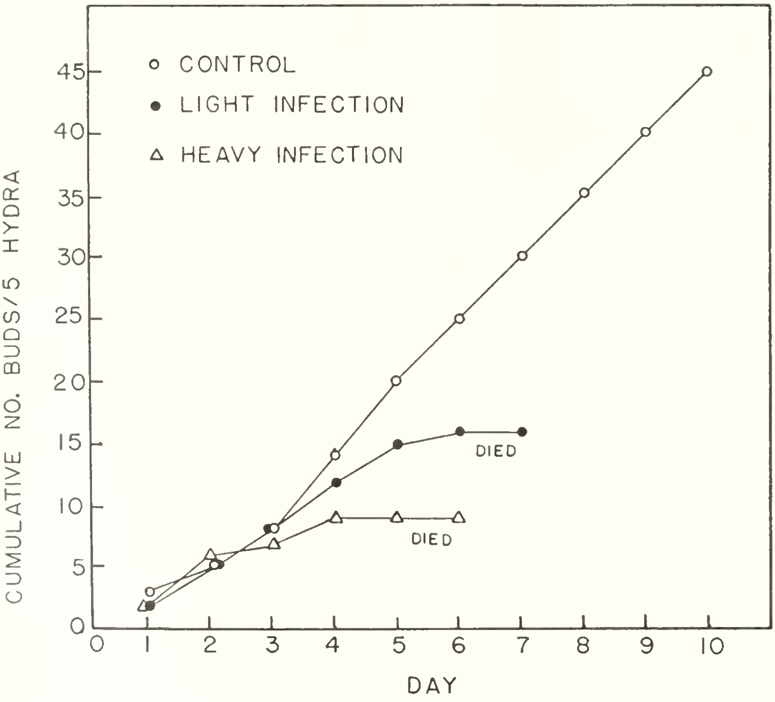


FIGURE 3. Total cumulative production of buds by 5 *Hydra pseudoligactis* under three levels of initial infection. Temperature was 25° C. The mean day of death occurred between the 5th and 6th day and the 6th and 7th day for heavily and lightly infected hosts, respectively.

Mechanisms contributing to reduction in budding

From the experiments employing *C. viridissima*, there is available a certain amount of information which may reveal the nature of the mechanisms leading to a differential decline and cessation of budding. When a host becomes infected, the hydramoeba population first increases to a certain level; next, it either stabilizes or fluctuates slightly; then, just prior to and during the disintegration of the host, a final increase occurs (Stiven, in press). During the attack the tentacles are first consumed. Their loss renders the hydra incapable of feeding, and thus budding ceases. If the infection is severe, death usually follows. In an attempt to supply quantitative support for these observations the following pertinent features were recorded during the experiments: day on which each host stopped feeding, the total number of days the host did not feed if it still survived to the end of the tenth day, the day on which each hydra produced its last bud, and the day of death (disintegration) of the host. These data, represented by averages ± the standard error for each treatment combination, are given in Table III. Not all replicates entered into the computation of the averages; the value in brackets indicates the pertinent number of replicates. These data apply to the light and heavy infection categories, since the hydras of the control group continued to feed and produce buds until the termination of the experiment. At 15° C. and at either level of infection, the hosts also continued to feed and produce buds throughout the experiment. In fact, at the light initial infection level three of the five hosts had lost the infection by the tenth day. It appears that at 15° C., *C. viridissima* is able to survive the attack of the hydramoebae. The data of Table III indicate that as temperature increases from 20° to 30°, there is a corresponding decrease in the length of time infected hosts feed, a decrease in the period of budding, and a reduction in their length of life. These results are consistent for both levels of initial infection.

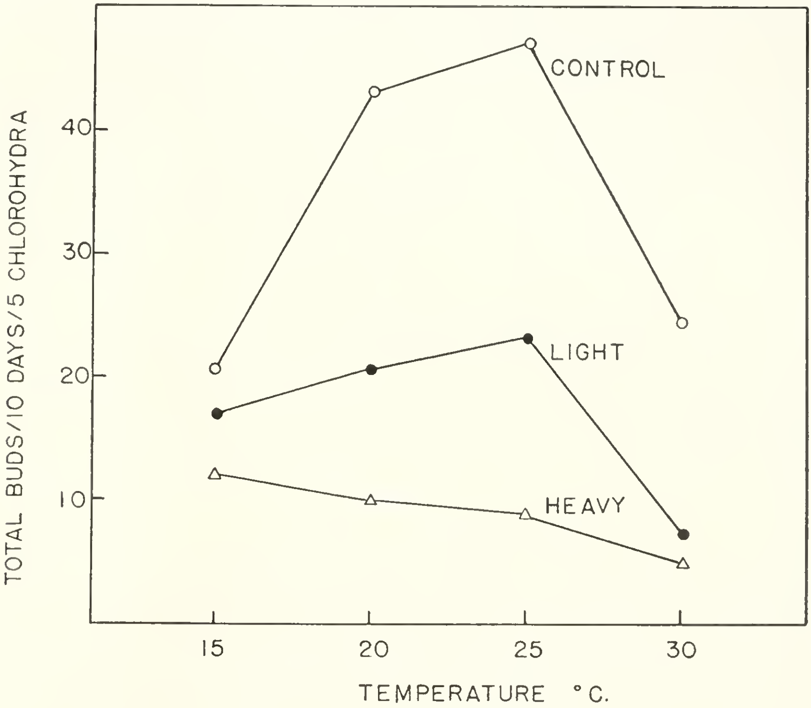


FIGURE 4. Illustration of the total number of buds produced in 10 days by groups of 5 *C. viridissima* under the various levels of temperature and initial infection. This depicts the significant interaction between temperature and infection and shows the lack of independence between these two factors.

Both the day the last bud was produced and the day of death of the host appear to be correlated with the day the host stopped feeding. Correlation coefficients were computed from the original data summing over all levels of temperature, excluding, of course, the 15°-level. In the first relationship $r = 0.67$ and in the latter $r = 0.78$ (both values are significant at $P < 0.01$), indicating a higher degree of correlation between the day the host stopped feeding and its eventual death. In this latter correlation, the regression coefficient is 1.23, meaning that if the host is able to feed 1.23 days longer, it can survive one more day, on the average. The regression coefficient of 0.68 in the first relationship means that an extra 0.68 day of feeding would yield an extra day of budding, on the average. Both regression coefficients are significant at $P < 0.05$.

Finally, considering budding rate in the control group, analysis of variance indicates that the mean number of buds produced in 10 days was significantly different among the levels of temperature (see Figure 4 also). Comparing each treatment mean with every other treatment by Duncan's multiple range test at the 5% level (see Steel and Torrie, 1960, p. 107) yielded the following result.

Temperature:	15°	30°	20°	25°
Mean no. buds/hydra/10 days:	4.2	4.8	8.6	9.4

The lines indicate that any two means underscored by the same line are not significantly different. Therefore, no difference in bud production exists between 15° and 30°, or between 20° and 25°. Differences do exist, however between 15° and 20°, 15° and 25°, 30° and 20°, and between 30° and 25°.

DISCUSSION

The effect of parasitism on individual hosts may take various forms, but what is frequently encountered is an inhibition or eventual destruction of the reproductive capacities of the host. Either can alter the population sex ratio, age structure, or be detrimental to the very survival of the population if the condition should persist for a substantial length of time. For example, Yoshida (1952) found that parasitic isopods which occupy the branchial chambers of the shrimp, *Leander*, inhibit the development of sexual activity in the shrimp. The cessation of production of normal gametes in crabs parasitized by the barnacle, *Sacculina*, is a well known example. In addition, larvae of strepsipteran insects frequently cause sterility in their homopteran hosts. Cheng and Snyder (1962) also cite several cases of the effect of larval trematodes on the gonads of several snail species. Nobel and Nobel (1961), in discussing more examples, point out that the mechanisms proposed to explain these effects on reproduction include the production of toxic substances, nutritional disturbances, hormone changes, and direct mechanical destruction of gonads.

The mechanism involved in the inhibition of asexual reproduction in hydra by the parasitic hydramoeba seems to be rather simple. It appears that hydramoebae first consume the tentacles of their host, thereby rendering them incapable of feeding. Since budding rate in hydra is in part a function of its nutritional state (Loomis, 1954; Stiven, 1962b), the obvious result of the attack of the hydramoebae is an inhibition and/or cessation of budding due to starvation, followed by the eventual death of the host.

The degree to which budding rate is inhibited, however, appears to be dependent upon the size of the initial infection. The size establishes the time at which the tentacles of the host are completely consumed, thus destroying the food-capturing ability of the host, and thereby inhibiting the host from continued cell proliferation and the formation of new buds. This conclusion is borne out by the significant correlation between the time the host stops budding and the day on which it stops feeding. Correlation coefficients, of course, measure only the degree of association between two variables and do not necessarily provide a cause and effect relationship. This must be decided by an examination of the biology of the relationship, which in this case, seems to provide a reasonable explanation for the high degree of correlation between cessation of feeding and cessation of budding.

The effect of the important environmental factor, temperature, was not independent of the action of initial parasitic infection beyond the first two cumulative days of the experiment. Figure 4 illustrates this for the 10-day period. For example, an increase of 5° (15° to 20° C.) increases bud production in the control group from 21 to 43, or 22 buds per five green hydras per 10 days. In the lightly infected group, the corresponding increase amounts to only four buds, whereas in the heavily infected group, a decrease of two buds occurs. From 25° to 30° C. decreases in total bud production occur at all levels of infection, but again the decreases are different. It is these differences that contribute to the significant interaction between temperature and infection. It must be emphasized also that the response of this host-parasite system, viewed through the interaction of these two factors, is dependent upon time. As indicated earlier, the interaction is not significant during the first two cumulative days of the experiment. Here the lines representing bud production between any two levels of temperature would be essentially parallel for the three levels of infection (*cf.* Fig. 4).

The importance of these results to experimental studies on the spread of the hydramoeba infection and to studies on factors contributing to declines in natural populations of hydra is probably best reflected in the response of the system to temperature. On the basis of the results reported here, it is postulated that at 15° C. an infected population of *C. viridissima* either eventually loses the infection and survives, other factors being favorable, or acquires some balance between the effects of the pathogenic hydramoebae and the reproductive capacity and survival of the host. In this latter case both host and parasite survive. In fact, since *Hydramoeba hydroxena* does not appear to have a viable cyst (Beers, 1963), such a relationship would constitute a reservoir of parasites. Long-term experiments are required before much credence can be given to this hypothesis.

As temperature increases and approaches 25° C., the pathogenic effects of the hydramoeba and the reproductive rate of the host both increase, but an instability develops in favor of the parasite, resulting in a cessation of budding and an eventual elimination of the host. Thus, the parasite population also succumbs. It is known that a temperature of 25° C. is very favorable for a rapid spread of the epidemic (Stiven, in press) and a subsequent rapid decline in the host population through death. The rapid elimination of the hydra population described by Bryden (1952) perhaps exemplifies this situation. Actually, temperatures around 30° C. are frequently encountered in surface waters of southern lakes in the late summer period (Weiss and Oglesby, 1962; Bryden, 1952), and these, combined with heavy infections of hydramoebae, would lead to an almost immediate cessation of budding and to a rapid death of the host. These high temperatures also lead to the production of large numbers of hydramoebae and probably explain their great abundance in late summer in Douglas Lake, as reported by Miller (1936).

SUMMARY

1. In prior studies on the spread of the parasite *Hydramoeba hydroxena* through populations of hydra, the density of host populations was kept fixed by removing buds and not allowing them to accumulate. Experimental analyses were undertaken, therefore, to determine the effect of this parasite on asexual reproduction in *Chlorohydra viridissima* under a range of temperatures from 15° to 30° C.

2. Budding rates were significantly different among light, heavy, and no (control) initial infections, and among four levels of temperature. The relationship between budding rate and temperature for the control group was curvilinear, with the highest rate occurring around 20° to 25° and the lowest around 15° and 30° C.

3. When the cumulative number of buds produced by the host was considered over the 10-day span of the experiments, it was found that temperature and initial infection were not independent of one another. This lack of independence was due to the fact that the difference in bud production among the levels of infection was not the same for the levels of temperature. The converse of this latter statement is also true.

4. *Hydra pseudoligactis,* which is less resistant to the attack of the hydramoebae, ceases budding almost immediately after infection.

5. In *C. viridissima,* significant correlations exist between the time the host stopped feeding and the day the host stopped budding, as well as between the time the host stopped feeding and the day it died from the attack of the hydramoebae. In addition, increases in temperature caused infected hosts to cease feeding sooner, a decrease in their period of budding, and a reduction in their length of life.

6. It appears that the destruction of the tentacles of the host by the hydramoebae renders the host incapable of feeding, and leads in turn to starvation and cessation of budding. The time at which the tentacles are rendered useless is dependent upon temperature and the size of the initial infection.

7. *C. viridissima* can tolerate the parasitic infection and continue budding at a lower temperature (*i.e.,* 15° C.). It is postulated that this balanced relationship may be a mechanism for providing a reservoir for *Hydramoeba hydroxena* in the absence of a viable cyst. However, this balance does not always occur at this temperature, since the infection is frequently lost. Long-term experiments are required before much weight can be given to this hypothesis.

LITERATURE CITED

BEERS, C. D., 1963. Lack of evidence for encystment in the parasitic amoeba *Hydramoeba hydroxena* (Entz). *J. Protozool.*, **10**: 495–501.

BRYDEN, R. R., 1952. Ecology of *Pelmatohydra oligactis* in Kirkpatricks Lake, Tennessee. *Ecol. Monogr.*, **22**: 45–68.

BURNETT, T., 1949. The effect of temperature on an insect host-parasite population. *Ecology*, **30**: 113–134.

CHENG, T. C., AND R. W. SNYDER, 1962. Studies on host-parasite relationships between larval trematodes and their hosts. I. A review. II. The utilization of the host's glycogen by the intramolluscan larvae of *Glypthelmins pennsylvaniensis* Cheng, and associated phenomena. *Trans. Amer. Micros. Soc.*, **81**: 209–228.

ENTZ, G., 1912. Über eine neue Amöbe auf Süsswasser-Polypen (*Hydra oligactis* Pall.). *Archiv. f. Protistenkunde*, **27**: 19–47.

ITO, T., 1949. On *Hydramoeba hydroxena* discovered in Japan. *Sci. Rep. Tohoku Univ., Ser. 4 (Biology)*, **18**: 205–209.

ITO, T., 1950. Further notes on *Hydramoeba hydroxena* (Entz) from Japan. *Mem. Ehime Univ., Sect. II (Science)*, **1**: 27–36.

LOOMIS, W. F., 1953. The cultivation of hydra under controlled conditions. *Science*, **117**: 565–566.

LOOMIS, W. F., 1954. Environmental factors controlling growth in hydra. *J. Exp. Zool.*, **126**: 223–234.

LOTKA, A. J., 1923. Contribution to quantitative parasitology. *J. Wash. Acad. Sci.*, **13**: 152–158.

MILLER, D. E., 1936. A limnological study of *Pelmatohydra* with special reference to their quantitative seasonal distribution. *Trans. Amer. Micros. Soc.*, **55**: 123–193.

NOBEL, E. R., AND C. A. NOBEL, 1961. Parasitology, the Biology of Animal Parasites. Lea and Febiger: Philadelphia.

REYNOLDS, B. D., AND J. B. LOOPER, 1928. Infection experiments with *Hydramoeba hydroxena* nov. gen. *J. Parasitol.*, **15**: 23–30.

RICE, N. E., 1960. *Hydramoeba hydroxena* (Entz), a parasite on the fresh water medusa, *Craspedacusta sowerbii* Lankester, and its pathogenicity for *Hydra cauliculata* Hyman. *J. Protozool.*, **7**: 151–156.

STEEL, R. G. D., AND J. H. TORRIE, 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., Inc.: New York.

STIVEN, A. E., 1962a. Experimental studies on the epidemiology of the host-parasite system hydra and *Hydramoeba hydroxena* (Entz). I. The effect of the parasite on the individual host. *Physiol. Zoöl.*, **35**: 166–178.

STIVEN, A. E., 1962b. The effect of temperature and feeding on the intrinsic rate of increase of three species of hydra. *Ecology*, **43**: 325–328.

STIVEN, A. E., in press. Experimental studies on the epidemiology of the host-parasite system hydra and *Hydramoeba hydroxena* (Entz). II. The components of a simple epidemic. *Ecol. Monogr.*, **34**.

WATT, K. E. F., 1959. A mathematical model of the effect of densities of attacked and attacking species on the number attacked. *Canad. Entomol.*, **91**: 129–144.

WEISS, C. M., AND R. T. OGLESBY, 1962. Limnology of a North Carolina reservoir and its use in water treatment. *J. Amer. Water Works Assoc.*, **54**: 671–683.

WELCH, P. S., AND H. A. LOOMIS, 1924. A limnological study of *Hydra oligactis* in Douglas Lake, Michigan. *Trans. Amer. Micros. Soc.*, **43**: 203–235.

YOSHIDA, M., 1952. On the breeding character of the shrimp, *Leander serrifer* parasitized by Bopyrids. *Annot. Zool. Japon.*, **25**: 362–365.

Vol. 126, No. 3 June, 1964

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE GENETICS OF ARTEMIA SALINA. IV. HYBRIDIZATION OF WILD POPULATIONS WITH MUTANT STOCKS[1]

SARANE THOMPSON BOWEN

Department of Biology, San Francisco State College, San Francisco, California 94132

The brine shrimp *Artemia salina* is a crustacean (Order Anostraca) which lives in the saline lakes and coastal salterns of five continents. (*Salterns* are ponds where sea water is concentrated by solar evaporation for the commercial production of sodium chloride.) Some populations are bisexual whereas others consist only of females which reproduce parthenogenetically. Some populations are diploid whereas others have been reported to be triploid, tetraploid, pentaploid, or octaploid on the basis of cytological studies. The morphological and cytological studies of *Artemia* populations have been reviewed by Stella (1933), Goldschmidt (1952), Barigozzi (1957), Dutrieu (1960), Gilchrist (1960), and Stefani (1961). The author knows of only two papers in which hybridization studies were mentioned. Gilchrist (1960, page 233) stated that North African shrimp and Californian shrimp would not interbreed. Barigozzi and Tosi (1959) mated Great Salt Lake shrimp to a diploid stock which evidently came from the Gulf of California.

The purpose of the present study was to determine whether nine wild populations of *Artemia* were reproductively isolated from one another. Evidence of reproductive isolation would be: (1) inability of the two populations to live in the same medium (habitat isolation), (2) failure of the male to clasp the female (ethological isolation), (3) failure to produce a viable F_1 (due to mechanical isolation, gametic or zygote mortality, or hybrid inviability), or (4) hybrid sterility (absence of an F_2 or production of a deficient F_2). These isolating mechanisms have been defined and discussed by Mayr (1963, pp. 91 to 109).

This paper describes a series of hybridizations in which wild-type shrimp were out-crossed either to wild shrimp from different localities or to inbred stocks homozygous for recessive mutant genes. It was found that the shrimp from Mono Lake and from Sète were reproductively isolated from each other and from the other seven populations. However, there was no barrier to gene exchange among

---

[1] This research was supported by grants from the National Science Foundation (NSF G-23863 and NSF GB-1316). I am indebted to three students who aided in these studies: Miss Jean Hanson, Mr. Denis Baskin, and Mr. Dan Straus.

Copyright © 1964, by the Marine Biological Laboratory

the other populations which therefore represent seven geographical races within a single species.

MATERIALS AND METHODS

Culture technique

The glassware, genetic techniques, and standard feeding schedule have been described in an earlier paper (Bowen, 1962). Mono Lake shrimp were cultured in water collected from the lake in May (sp. gr. of 1.046). The other wild races, the mutant stocks, and the progeny of experimental matings were cultured in the *standard medium* (50 grams of NaCl per liter of filtered sea water). The females are viviparous when cultured in this medium and fed yeast according to the standard feeding schedule. They do not produce dormant cysts unless the medium is modified by the addition of monosodium iron (ferric) ethylenediaminetetraacetic acid.

Females were isolated at least two weeks before they were mated to males from another race or stock. Genetic experiments have shown that females do not store sperm from one reproductive cycle to the next (Bowen, 1962). All matings described in this paper were single-pair matings in shell vials containing 5 ml. of the standard medium. Every week, the matings were checked and if nauplii were present, they were transferred to other shell vials (2–3 nauplii per vial). All shrimp were routinely examined without anesthetization under a dissecting microscope (7 ×) once per week.

Source of the wild populations

Sète. Cysts of this parthenogenetic race from salterns on the Mediterranean at Sète, France, were obtained through the courtesy of Dr. Barbara M. Gilchrist. The Sète stock is descended from a single female hatched from a cyst in 1961.

San José Island. This stock is descended from ten adults collected by Jean Hanson from the salterns on this island in the Gulf of California near La Paz, Baja California Sur, Mexico.

Little Manitou Lake. Cysts from this lake in Saskatchewan, Canada were obtained through the courtesy of Mr. R. P. Dempster, President of the San Francisco Aquarium Society.

Quemado. Cysts were collected by Mr. Thomas D. Foster from the salt lake near Quemado, New Mexico, U. S. A.

Great Salt Lake. The shrimp used in the racial crosses came from four samples of cysts collected in different years from this lake in Utah, U. S. A. Two collections were made by Mr. C. C. Sanders, one by Mr. M. Rakowicz, and one by Dr. J. S. Hensill.

San Diego. Cysts were collected by Mr. D. M. Miller, an executive of Western Salt Company, from salterns on South San Diego Bay, California, U. S. A.

Moss Landing. Our stock is descended from two adults collected by Miss June Akiyoshi from salterns on Monterey Bay, California, U. S. A.

San Francisco. The shrimp used in the racial crosses were derived from four samples of cysts collected in different years from salterns of the Leslie Salt Company at the south end of San Francisco Bay, California, U. S. A. Three collections were made by the author or her students and one collection was obtained from Mr. M. Rakowicz, President of Brine Shrimp Sales Co.

Mono Lake. Adults from this lake in California, U. S. A., were collected by the author in 1961 and by Dr. Joel Gustafson in 1963.

Origin of the mutant stocks

Stock #1. This inbred line is descended from shrimp from Great Salt Lake and is homozygous for the recessive gene, *r*, which determines red eyes (Bowen, 1962). This stock has been carried through more than 40 generations in the laboratory.

Stock #9. The origin of this stock has been described earlier (Bowen, 1963). It is derived from both the San Francisco and Great Salt Lake populations and consists of white-eyed males and pigmented-eyed (red or black) females. White eyes is determined by a recessive mutant gene, *w*. The white locus is located on the homologous portion of the sex chromosomes. In the #9 stock, crossing over between the white locus and the sex locus is suppressed. Therefore, this stock breeds true for X^wX^w males and X^wY^+ females.

RESULTS

1. *The Mono Lake population*

Mono Lake shrimp were cultured through three generations in the laboratory in Mono Lake water. However, in each of four independent experiments, ten pairs of Mono Lake shrimp died within one week after transfer to the standard culture medium. Similar attempts to culture Great Salt Lake and San Francisco shrimp in water from Mono Lake were unsuccessful. In another series of experiments, shrimp from Mono Lake and from San Francisco salterns were placed in solutions which combined varying amounts of standard medium, Mono Lake water, or saltern water. Every combination supported one population; no combination was satisfactory for rearing both populations. Therefore, hybridization experiments could not be carried out with shrimp from Mono Lake.

2. *Attempts to mate mutant males with parthenogenetic females*

The Sète stock has been carried through 15 generations in this laboratory. More than 1000 offspring have been reared to maturity; all were parthenogenetic females.

The reproductive cycle of the *Artemia* female has been described by many authors. The viviparous female expels from the uterus a first brood of nauplii. The next brood of eggs passes from the ovaries into the oviducts where they remain for about one day. At this time they are in metaphase of the first meiotic division. They pass next into the uterus where segmentation occurs. Finally they are expelled into the culture medium as the second brood of nauplii. In the Sète population, the eggs develop without fertilization. In the Utah bisexual population, copulation must occur at the time the eggs are in the oviduct if fertilization is to occur (Bowen, 1962).

A series of single-pair matings was made: each consisted of a wild-type (black-eyed) Sète female and either a red-eyed or white-eyed male (from stock #1 or #9). Daily observations were made of clasping and attempted copulation in relation to the female reproductive cycle. Forty-one broods of nauplii were obtained; all matured into wild-type females. Eleven of these broods came from matings in which the male was seen to be clasping and attempting to copulate at the time the eggs were in the oviducts. These 11 broods consisted of 112 wild-type female shrimp. Although they were reared in the absence of males, they produced a second generation of offspring of which 176 shrimp were classified and found to be wild-type females.

If fertilization had occurred in the original matings, one would expect that the sperm would affect development of the parthenogenetic egg in such a way as to produce an abnormal F_1 (consisting of intersexes, or sterile triploids, or shrimp bearing patches of mutant tissue of androgenetic origin). However, all of the first and second generation progeny were wild-type parthenogenetic females. We can conclude that although males will clasp and attempt to copulate with Sète females, the sperm do not affect the development of the Sète embryo.

3. *Differences in morphology of wild populations*

When reared under identical environmental conditions, some wild-type populations of *Artemia* are morphologically distinguishable. Gilchrist (1960) has shown that if shrimp of the same total length are compared, females of the California (San Diego) stock have a shorter and broader abdomen than females of the Algerian stock. The two populations were reared under identical conditions in order to rule out the effect of salinity on body form (reviewed by Gilchrist, 1959, 1960). The present author has also observed quantitative differences in body dimensions in certain races grown for two or more generations under standard

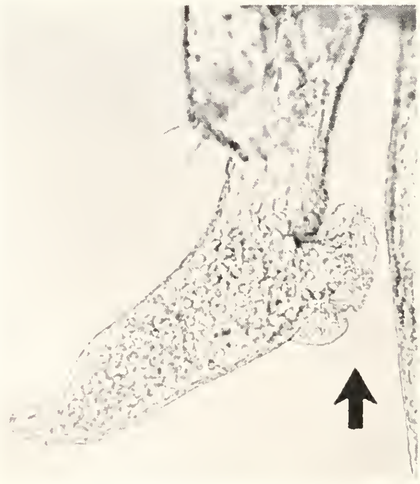


FIGURE 1. Antenna of wild-type female from Quemado, New Mexico. The three-lobed projection (arrow) on the posterior surface is present in females of this race only. Photograph of living animal (lateral aspect, 60 ×).

TABLE I

Presence of antennal projection

	Females number with projection / total number examined	Males number with projection / total number examined
Great Salt Lake	0/10	0/10
San Francisco	0/10	0/10
Quemado	10/10	0/10
F_1 from matings of Quemado ♂♂ to San Francisco ♀♀	11/12	0/7
Progeny of F_1 ♂♂ × San Francisco ♀♀	24/41	0/23
Progeny of F_1 ♀♀ × San Francisco ♂♂	8/21	0/13

laboratory conditions. Only one qualitative morphological difference has been found: an antennal structure present in females from the Quemado race but absent in all other wild shrimp. This structure and its mode of inheritance will now be described.

The head of the brine shrimp bears a pair of slender unjointed *antennules* and a larger pair of *antennae*. The antennae show sexual dimorphism, being greatly enlarged and modified for clasping in the male. In the Quemado race, the females develop a *projection* on the posterior surface of the antenna, located about one-third of the distance from the distal tip. In young sexually mature females (four weeks old), the projection is a small spike. In older females, the projection has a broader base and terminates in two or three lumps (Fig. 1). No similar structure could be found on the antennae of females from Utah or San Francisco or of males from Great Salt Lake, San Francisco, or Quemado (Table I). This trait was of interest because of the possibility that it might be determined by genes on the non-homologous portion of the Y chromosome. The female is the heterogametic sex in *Artemia* and has the XY chromosome constitution (Bowen, 1963).

Four matings of Quemado males to San Francisco females were fertile. Some F_1 progeny from each mating were backcrossed to San Francisco shrimp. Because of the time required to examine the antennae under high power magnification (160 ×), only a small sample of the F_1 and backcross progeny were examined for the presence of the projection. More females were examined because it was evident that males could not have the projection (see the data in the last three rows in Table I). Note that most of the F_1 females have the projection. This indicates that the Quemado males can transmit this trait to their daughters. Both male and female F_1 hybrids can transmit the trait to their daughters. We may conclude that this projection is determined by autosomal dominant genes at one or more loci. These genes are sex-limited; that is, they are present in both sexes but have no effect upon the phenotype of the male.

In the preceding discussion, the author has assumed that the projection on the Quemado female antenna is not homologous with the *frontal knob,* a disc-like projection on the basal segment of the antennae of males from all *Artemia* populations. However, even if these two structures were homologous, we would again conclude that the projection-knob trait is governed by autosomal loci.

4. *Hybridization among seven bisexual North American populations*

The seven populations which were successfully hybridized were from San José Island (Mexico), Little Manitou Lake (Canada), and from five localities in the U.S.A.: Great Salt Lake, Quemado, San Francisco, Moss Landing, and San Diego. Evidence has been presented above (Table I) that matings of Quemado males to San Francisco females gave progeny which were fertile when backcrossed to San Francisco shrimp. In a second series of matings, wild-type shrimp were mated to wild shrimp from other localities and the progeny were mated *inter se* to produce an F_2 (Table II). In a third series of matings, reciprocal crosses were made of each of the seven populations to shrimp from stock #1 which is homozygous for the recessive mutant gene for red eyes. The F_1 progeny were mated *inter se* to produce an F_2 (Table III). The experiments summarized in Tables I and III provide a rigorous proof of hybridization because the recovery of the genetic markers (antennal projection and red eyes, respectively) rules out the possibility of parthenogenetic or pseudogamic reproduction or experimental errors such as the use of non-virgin females in the outcrosses.

The experiments summarized in Tables II and III were designed in such a way that an F_1 and F_2 would be obtained from every fertile parental pair. For example, in Table III nine matings were set up in which males from the mutant stock were mated to females hatched from cysts collected from Great Salt Lake. Each of the eight fertile pairs produced at least one F_1 brood which when mated *inter se* gave rise to a normal F_2 brood.

In Tables II and III, the source of the wild parents (either cysts collected from the natural habitat or inbred laboratory stocks) is stated to enable the reader to determine the number of independent genotypes which were sampled from each

TABLE II

Hybridization of six wild populations of brine shrimp

Parental cross Male × Female	Source of parents	Parental crosses fertile pairs / total matings	Number of F_1 hybrids reared to maturity	Normal F_2 progeny obtained from each fertile parental pair
Gt. Salt Lake × Gt. Salt Lake (control)	cysts	6/8	116	
San Francisco × San Francisco (control)	cysts	9/13	176	
San Francisco × Gt. Salt Lake	cysts	2/6	83	yes
Gt. Salt Lake × San Francisco	cysts	1/3	113	yes
Little Manitou × Quemado	cysts	2/2	130	yes
Quemado × Little Manitou	cysts	1/2	17	yes
San José Is. × Gt. Salt Lake	inbred stocks	3/3	110	yes
San José Is. × San Francisco	inbred stocks	2/2	25	yes
San Francisco × Moss Landing	inbred stocks	1/1	5	yes
Little Manitou × San Francisco	cysts	1/1	28	yes

TABLE III

Results of mating seven races of wild brine shrimp to stock #1 which is homozygous for the recessive gene for red eyes

Parental cross Male × Female	Source of wild parents	Parental crosses fertile pairs / total matings	Number of F_1 hybrids reared to maturity (wild phenotype)	Number of F_2 progeny reared to maturity	Red-eyed shrimp found in F_2 from every fertile parental pair
Gt. Salt Lake × stock #1	cysts	5/8	283	94	yes
stock #1 × Gt. Salt Lake	cysts	8/9	606	421	yes
San Francisco × stock #1	cysts	3/4	342	117	yes
stock #1 × San Francisco	cyst	1/1	61	227	yes
Quemado × stock #1	cysts	6/7	329	93	yes
stock #1 × Quemado	cysts	3/4	291	261	yes
Little Manitou × stock #1	inbred stock	4/5	130	63	yes
stock #1 × Little Manitou	inbred stock	2/5	190	63	yes
Moss Landing × stock #1	inbred stock	1/1	6	9	yes
stock #1 × Moss Landing	inbred stock	1/1	14	67	yes
San Diego × stock #1	cysts	2/3	68	61	yes
stock #1 × San Diego	cysts	1/3	9	8	yes
San José Is. × stock #1	inbred stock	4/4	155	36	yes
stock #1 × San José Is.	inbred stock	1/1	16	14	yes

population. The next column in both tables gives the fraction of successful (fertile) matings. This is primarily an indication of the efficiency of the laboratory technique. That is, the failure of a mating may be due to the death or poor health of one parent rather than to reproductive isolation of a genetic nature. Evidence supporting this interpretation comes from the observation that only 9/13 of the control matings, San Francisco ♂♂ × San Francisco ♀♀, were successful (Table II). In another study of 46 matings of stock #1 ♂♂ × stock #1 ♀♀, at the end of a two-week period only 31 females were alive and of these only 16 had produced progeny.

Although a few F_1 nauplii from each hybridization were examined carefully under the high-power microscope (120 ×), no abnormal hybrids were found of the type described by Barigozzi and Tosi (1959). They stated that when Great Salt Lake *Artemia* were crossed to a diploid race, the F_1 offspring were triploids of abnormal appearance: stunted body and enlarged lateral eyes and limbs.

The experiments summarized in Tables II and III were designed to sample the F_1 and F_2 generations from the greatest number of parental crosses. In order to speed the sampling process, many broods were discarded before the last shrimp in the brood reached the stage of sexual differentiation. (There is great variation in growth rates, even in highly inbred lines of *Artemia.*) For this reason, sex ratios and genetic segregation data are not given in either table. However, aberrant red eye segregation ratios and aberrant sex ratios were not noted. At weekly intervals, the number alive in each brood was recorded. The viability of the hybrids did not differ significantly from that of the controls (San Francisco wild-type nauplii). Forty-nine per cent (220/450) of the San Francisco shrimp were alive at the end of three weeks.

Three F_2 broods from parental crosses of stock #1 × Great Salt Lake and four F_2 broods from crosses of stock #1 × San Francisco were reared to maturity and their eyes were examined at 4-day intervals. In order to classify eye color correctly, each shrimp must be isolated and examined throughout development because some heterozygotes have ruby eyes for a brief period (Bowen, 1962). The combined values from the seven broods were: 34 with red eyes and 115 with black eyes or 34/149 (23%) with r/r genotype. This is in good agreement with the value of 25% expected in the F_2 progeny from a cross of two parental diploids.

The sex-ratio data in Table IV are taken from some of the experiments summarized in Tables II and III and from additional matings of wild-type populations with mutant stocks. The ratios do not show significant deviations from the expected value of 50% males.

TABLE IV

Sex ratios in F_1 progeny of crosses of wild-type brine shrimp to mutant stocks

Parental cross Male × Female	F_1 hybrids		Per cent males
	Males	Females	
Gt. Salt Lake × mutant stock	178	166	52%
mutant stock × Gt. Salt Lake	120	132	48
San Francisco × mutant stock	148	140	51
mutant stock × San Francisco	196	200	50
Quemado × mutant stock	106	119	47
mutant stock × Quemado	130	144	47
Total	878	901	49

DISCUSSION

The parthenogenetic population

When males from mutant stocks were mated to parthenogenetic females from Sète, hybrid offspring were not obtained (see part 2). These negative results suggest that males cannot introduce genes into the parthenogenetic population. The Sète population consists of a cluster of clones, each of which is reproductively isolated from every other clone and from males of other localities. Each Sète female is a genetic isolate.

The Mono Lake population

Hybridization experiments could not be made with the shrimp from Mono Lake because they have different physiological requirements; they die when transferred into media in which the other populations were cultured (see part 1). These negative results suggest that the Mono Lake bisexual population cannot exchange genes with the other seven bisexual populations. It is reproductively isolated due to habitat selection. Because it is morphologically similar to other populations of *Artemia* (see part 3), it represents a sibling species.

The seven geographical races

The other seven bisexual populations (from San José, Little Manitou, Great Salt Lake, Quemado, San Francisco, Moss Landing, and San Diego) are not reproductively isolated and therefore represent geographical races within a single species. Evidence for this is the fact that they produce hybrids with normal viability and fertility. Although the wild populations were not crossed in every possible combination, it was evident that shrimp from the ends of the Pacific Coast distribution of salterns (San Francisco and San José Island) were cross-fertile with each other and with the Great Salt Lake race. Similarly, the shrimp from the most northern and southern inland lakes (Little Manitou and Quemado) were cross-fertile with each other and with the San Francisco race (Table II).

In a second series of matings, reciprocal crosses were made of each race with shrimp from a mutant stock homozygous for the recessive gene for red eyes (Table III). The F_1 progeny had normal morphology, viability, and fertility and the mutant phenotype was seen again in the F_2. The sex ratios in the F_1 generation of each cross did not show significant deviation from the expected value of 50% males (part 4 and Table IV).

There is no intrinsic barrier to gene exchange among these races. Genes from one race might be introduced into another if cysts were carried into a new habitat on the legs or in the digestive tracts of migrating birds. However, geographical isolation has been sufficiently effective to bring about the divergence of the Quemado population in regard to the morphology of the antenna (described in part 3).

Ploidy of the seven bisexual populations

The seven races listed in Tables II and III must be at the same level of ploidy; *i.e.*, all seven must be diploid or all seven must be tetraploid. For, if a diploid were crossed to a tetraploid, the hybrid offspring certainly would be sterile triploids, due to irregular meiosis, and possibly the sex ratio would be altered.

Nakanishi, Okigaki, Kato and Iwasaki (1963) made chromosome counts on somatic cells of nauplii hatched from cysts obtained from the San Francisco Aquarium Society. Cysts distributed under the Society name are collected by Brine Shrimp Sales Company of Hayward, California, from the salterns of the Leslie Salt Company on San Francisco Bay (personal communication from Mr. James A. Mason, General Manager of Brine Shrimp Sales Company). Nakanishi *et al.* reported that the chromosome number ranged from 16 to 48 but there was a distinct peak formed by cells showing 42 chromosomes. If the San Francisco race is a diploid ($2n = 42$), we must conclude that the six races which produce fertile hybrids when crossed to this race are also diploid. This line of reasoning indicates that the seven cross-fertile races are diploid.

This conclusion is not in agreement with the statement of Metalli, Ballardin and Barigozzi (1961) that the race from San Francisco Bay was "predominantly diploid" whereas the race from Great Salt Lake was "predominantly tetraploid" (pages 410 and 417 of their 1961 paper). Barigozzi and Tosi based their decision that the Utah shrimp were tetraploid ($4n = 84$) on cytological studies and on the results of a hybridization experiment. Seven matings were made of Great Salt Lake shrimp to animals from a diploid stock. The hybrid progeny were abnormal in appearance and died before reaching maturity (Barigozzi and Tosi, 1959). It is difficult to evaluate these negative results because the authors did not state their culture method or give an estimate of the viability of non-hybrid nauplii. Somatic chromosome counts on the abnormal hybrids (presumed triploids) gave values in the range from 32 to 63. Additional evidence for tetraploidy came from chromosome counts on male germ cells, nauplius somatic cells, and primary oocytes of the Great Salt Lake race (Barigozzi and Tosi, 1957, 1959). When oocytes of Utah females were examined, the metaphase-I plates consisted in some cases of "42 isolated tetrads" while in other plates these tetrads were "ordered two by two, corresponding to 21 tetravalent bodies" (page 3 of their 1959 paper). The genetic experiments reported in the present paper suggest that the 21 "tetravalent" elements may have been 21 tetrads, *i.e.*, 21 bivalents. In the cytological studies of parthenogenetic *Artemia*, there have been disagreements among several authors in regard to the interpretation of the elements seen on the metaphase-I spindle of several races (reviewed by Goldschmidt, 1952). For example, the Kalia race was first reported to be a decaploid because the 107–109 elements were interpreted as tetrads (bivalents). In a later study, they were interpreted as univalents and the race was reported to be pentaploid (Haas and Goldschmidt, 1946; Goldschmidt, 1952).

Several explanations might be advanced for this lack of agreement between the conclusions in the present paper and those of Dr. Barigozzi and his co-workers in regard to the ploidy of the Utah shrimp. One possibility might be the use of wild shrimp of mistaken origin. However, in the studies reported here, the shrimp from Great Salt Lake, Utah, were hatched from four samples of cysts which were collected and handled independently. Readers who are acquainted with the earlier literature of *Artemia* might wonder whether the progeny obtained from outcrossed Utah females might be explained by parthenogenesis in the Utah population. (Many textbooks state that the Utah race is parthenogenetic.) However, parthenogenesis has never been observed in the seven North American races maintained in this laboratory (those listed in Table III). Extensive genetic tests for pseudogamic and parthenogenetic reproduction were conducted on Utah shrimp without positive results (Bowen, 1962). There are two more probable explanations of the disagreement. First, some cysts from Great Salt Lake may indeed be tetraploid. These may have given rise to the stock used by Dr. Barigozzi. In that case, we would conclude that Great Salt Lake contains two reproductively isolated sympatric populations: one diploid and one tetraploid. Such a situation would not be stable for long since every $4n \times 2n$ mating would result in the elimination of an equal number of gametes from each population. This process would eventually eliminate the population which had the smaller initial numbers. Another possibility is that the Great Salt Lake race is entirely diploid. In that case, the inviable progeny obtained in the hybridization experiments of Barigozzi and Tosi (1959) might have resulted from the chance selection of seven females which would have given birth to inviable progeny regardless of the origin of their mates. Some matings consistently produce thin-shelled eggs which soon decompose or broods of nauplii which die before reaching sexual maturity (Bowen, 1962, p. 27, and Part 4 of this paper).

Evidence has been presented that the Great Salt Lake race of *Artemia* is cross-fertile with six other North American races: San José Island, San Diego, Moss Landing, San Francisco, Little Manitou, and Quemado. Sixteen shrimp, each hatched from an encysted blastula collected from the shores of Great Salt Lake, were crossed to San Francisco shrimp or to a mutant laboratory stock known to be cross-fertile with the other six races (Tables II and III). The F_1 males and females from all sixteen matings were normal in appearance and fertile. In thirteen of these matings, a genetic marker was used and the recessive mutant trait was recovered in the F_2 generation (Table III). Genetic segregation ratios were recorded from some of these matings and the data did not deviate significantly from the values expected in the F_2 of two parental diploids (see part 4). The F_1 sex ratios did not deviate significantly from the expected 1:1 ratio of males to females (Table IV).

SUMMARY

Brine shrimp were collected from eight bisexual populations in North America and from one parthenogenetic population in Sète, France.

1. Males from mutant bisexual stocks were mated to wild-type Sète females. The progeny were wild-type females which parthenogenetically produced a second all-female generation. Although males will clasp and attempt to copulate with Sète females, their sperm do not affect the development of the parthenogenetic egg.

2. Hybridization experiments could not be carried out with the bisexual shrimp from Mono Lake because they died when transferred into the medium in which the other eight populations were cultured. This suggests that the Mono Lake population is reproductively isolated from the others.

3. A projection found only on antennae of females from Quemado, New Mexico, is determined by dominant autosomal genes with sex-limited expression.

4. There was no evidence of reproductive isolation among the *Artemia* from seven North American localities: San Francisco, Great Salt Lake, San Diego, Moss Landing, San José Island, Little Manitou, and Quemado. Reciprocal crosses were made of shrimp from each of these seven populations with shrimp from a mutant stock which was homozygous for the recessive gene for red eyes. In all crosses, the F_1 progeny had normal morphology and viability and the expected 50:50 sex ratio was obtained. The F_1 progeny were fertile and the mutant phenotype was recovered in the F_2 generation. Therefore, these seven populations have no intrinsic barrier to gene exchange and represent geographical races within a single species. Because cytologists agree that the San Francisco race is diploid, it is assumed that the six races with which it is cross-fertile are also diploid. This statement is not in agreement with the conclusion of Barigozzi and Tosi (1959) that the Great Salt Lake *Artemia* are tetraploid.

LITERATURE CITED

Barigozzi, C., 1957. Différenciation des génotypes et distribution géographique d'*Artemia salina* Leach: données et problèms. *Année Biol.*, **33**: 241–251.

Barigozzi, C., and M. Tosi, 1957. Nuovi dati sul numero cromosomico di *Artemia salina* anfigonica. *La Ricerca Scientifica*, suppl. III Riunione AGI, **27**: 3–5.

Barigozzi, C., and M. Tosi, 1959. New data on tetraploidy of amphigonic *A. salina* Leach and on triploids resulting from crosses between tetraploids and diploids. Convegno di Genetica, 1957. *La Ricerca Scientifica*, Suppl., **29**: 3–6.

Bowen, S. T., 1962. The genetics of *Artemia salina*. I. The reproductive cycle. *Biol. Bull.*, **122**: 25–32.

Bowen, S. T., 1963. The genetics of *Artemia salina*. II. White, a sex-linked mutation. *Biol. Bull.*, **124**: 17–23.

Dutrieu, J., 1960. Observations biochimiques et physiologiques sur le développement d'*Artemia salina* Leach. *Arch. de Zool. Exp. Gén.*, **99**: 1–133.

Gilchrist, B. M., 1959. The metabolism of *Artemia salina* (L.). Ph.D. thesis, University of London.

Gilchrist, B. M., 1960. Growth and form of the brine shrimp *Artemia salina* (L.). *Proc. Zool. Soc. Lond.*, **134**: 221–235.

Goldschmidt, E., 1952. Fluctuation in chromosome number in *Artemia salina*. *J. Morph.*, **91**: 111–133.

Haas, G., and E. Goldschmidt, 1946. A decaploid strain of *Artemia salina*. *Nature*, **158**: 239.

Mayr, E., 1963. Animal Species and Evolution. The Belknap Press of Harvard University Press, Cambridge, Mass.

Metalli, P., E. Ballardin and C. Barigozzi, 1961. Primi risultati del trattamento con raggi X di *Artemia salina* Leach. *Atti Assoz. Genet. Ital.*, **6**: 409–418.

Nakanishi, Y. H., T. Okigaki, H. Kato and T. Iwasaki, 1963. Cytological studies of *Artemia salina*. II. Deoxyribonucleic acid (DNA) content and the chromosomes in encysted dry eggs and nauplii. *Proc. Japan Acad.*, **39**: 306–309.

Stefani, R., 1961. Differenze nel ciclo annuale tra biotipo anfigonico e biotipo partenogenetico nell'*Artemia salina* di Cagliari. *Rivista di Biologica*, **54**: 457–469.

Stella, E., 1933. Phaenotipical characteristics and geographical distribution of several biotypes of *Artemia salina* L. *Zeitschr. indukt. Abstamm.-u. VererbLehre*, **65**: 412–446.

White, M. J. D., 1946. The evidence against polyploidy in sexually reproducing animals. *Amer. Nat.*, **80**: 610–618.

THE EFFECT OF NUTRIENT MEDIA UPON HEAD FREQUENCY IN REGENERATING PLANARIA[1]

S. J. COWARD, R. A. FLICKINGER AND E. GAREN[2]

Department of Zoology, University of California, Davis, California

The term "head frequency," as used by Child (1941), refers to the completeness and rate of differentiation of head structures in regenerating planaria. In most species of planarians the regeneration of head structures is less complete and occurs more slowly at successively more posterior levels. Heads regenerated at progressively posterior levels are smaller, until no head regeneration at all occurs at the extreme caudal end of the worm. There is also an anterior-posterior gradient of rate of regeneration of head structures, and this can be visualized most easily by comparing the time required after cutting the worms for the formation of eyespots at different levels of the worm.

Quantitative anterior-posterior gradients of incorporation of C^{14}-labeled CO_2 and glycine into the protein fraction of planaria (*Dugesia tigrina* and *D. dorotocephala*) have been demonstrated (Flickinger, 1959). These gradients can be abolished by treating the worms with chloramphenicol or colcemide (deacetylmethylcolchicine) for one or two days. Furthermore, the polarity of regenerating worms can be reversed by embedding the cut pieces in agar slabs and then immersing the prospective anterior end of the worm into a solution of chloramphenicol or colcemide (Flickinger, 1959; Flickinger and Coward, 1962). Presumably the gradient has been altered by this treatment, inhibiting the prospective head end of the cut piece, thus allowing cephalic differentiation at the prospective caudal end.

These experiments illustrate that reversal of the normal gradient of protein synthesis reverses the polarity of the regenerating worm, but such experimental control of polarity is of a negative nature. On the other hand, Brønsted and Brønsted (1953) have shown that addition of yeast sodium ribonucleate to starved decapitated planaria can accelerate the time of eyespot formation, which suggests that certain metabolites playing a part in protein synthesis are not present at optimal levels in starved planaria. It is known that the head frequency (*i.e.,* extent and rate of head formation) is greatest in anterior regions, most easily evidenced by the fact that eyespots form later in the posterior regions. The results of Brønsted and Brønsted (1953) suggested that treatment of sections from different body levels with sodium ribonucleate, amino acids, or other substances that might promote protein synthesis, would alter the head frequency gradient.

MATERIALS AND METHODS

The planaria, *Dugesia dorotocephala,* were obtained from a commercial supplier and were starved for various periods of time before being used. Before

---

[1] This investigation was supported by grants from the National Science Foundation (G-19963) and a University of California Cancer Research Grant.

[2] Mr. Garen was supported by a National Science Foundation summer training program in biology for high school students.

cutting, the worms were washed several times with boiled tap water, exposed to bacteriocidal ultraviolet light for five minutes and then cut with cataract knives. The heads and tails were removed and the rest of the worm was cut into three or six pieces of equal size (Fig. 1). In tabulating the results the pieces are numbered in an anterior-posterior direction. In some experiments with the worms cut into six pieces, the middle pieces (3 and 4) containing the pharynx were not used. In those worms cut into three parts, the pieces corresponding to pieces 1 + 2, 3 + 4 and 5 + 6 of the worms divided into six parts. The horse serum, chick embryo extract, vitamin and amino acid mixtures (essential and non-essential amino acids of Eagle's basal medium) were obtained commercially; the horse serum and chick embryo extract were shipped in the frozen state. A penicillin-streptomycin mixture (1%) was present in each of the media, including the boiled tap water.

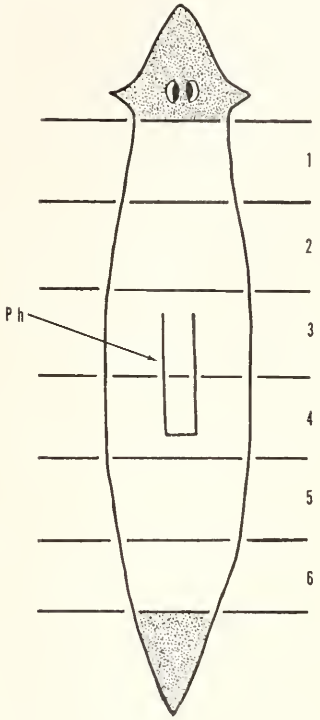


Figure 1. Diagram showing the regions used for regeneration experiments after removal of the head and tail. The pieces (3, 4) containing the pharynx (Ph) were not used in some of the experiments.

In the first type of experiment the compounds to be tested were dissolved together in a 1/10 dilution of Niu-Twitty saline (Niu and Twitty, 1953) to provide a complex nutrient medium. In the other experiments these fractions were dissolved in Seitz-filtered tap water and tested individually. In two series of experiments with yeast sodium ribonucleate, cut sections were exposed only to sodium RNA for one day after cutting, subsequently being transferred to boiled tap water.

In all other experiments the cut worms were maintained continually in the test medium or boiled tap water in small Stender dishes. The pH's of the various media were 7.2 to 8.0. The regenerating worms were maintained at 20° C. and transferred to fresh media every second day.

RESULTS

Experiments with complex nutrient medium; continual exposure

The heads and tails of 30 worms that had been starved for 5 days were cut away and the remaining midsection cut into three pieces of equal size. The cut sections from 15 of these worms were cultured in boiled tap water containing 1% penicillin-streptomycin; the cut pieces of 15 other worms were cultured in a 1/10 dilution of Niu-Twitty medium containing 5% horse serum, 1% chick embryo extract, 1% vitamin mixture, 0.5% concentration of both the essential and non-essential amino acid mixtures of Eagle's medium, 1% glutamine, 0.2% glucose and 1% penicillin-streptomycin under a 95% air–5% CO_2 atmosphere. Observation of these cut worms revealed that the head frequency was higher in the anterior (number 1) as compared to the middle (number 2) and posterior (number 3) sections. However, the nutrient medium had no significant effect in accelerating the time of eyespot appearance or the number of eyespots differentiating at any of the three levels.

TABLE I

Regeneration of eyespots in nutrient medium versus tap water; worms starved 10 days

Medium	Level of section*	Number of sections	Number with eyespots			
			5	6	7	8
			Days after cutting			
A. Tap water	1 + 2	15	10	13	15	15
B. Nutrient medium**	1 + 2	14	11	14	14	14
A. Tap water	3 + 4	13	1	2	5	6
B. Nutrient medium	3 + 4	15	9	13	14	14
A. Tap water	5 + 6	13	1	6	7	7
B. Nutrient medium	5 + 6	15	7	12	14	14

* The numbers refer to the various levels shown in Figure 1.

** The nutrient medium was composed of 5% horse serum, 0.5% amino acids, 1% chick embryo extract, 1% vitamin mixture and 0.2% glucose in sterile tap water.

This experiment was then repeated with worms that had been starved for 10 days instead of 5 days. In this experiment both the extent and time of appearance of eyespots in the middle and posterior pieces (levels 2 and 3) were stimulated in those regenerates maintained in the nutrient medium, as compared to the controls kept in tap water, but there was no effect upon the anterior pieces (level 1)

(Table I). Six days after cutting, 13 of the 15 cut midsections in the nutrient medium had formed eyespots, while only 2 of 13 in tap water had formed eyespots; 12 of 15 of the posterior pieces in nutrient had formed eyespots, *versus* 6 of 13 pieces in tap water. Thus, the frequency of eyespot formation, as well as the rate, is influenced by the nutrient medium. Eight days after cutting of the most posterior sections (level 3), 14 of 15 pieces in the nutrient medium possessed eyespots; at this same time eyespots were present in only 7 of 13 pieces that regenerated in tap water. Subsequent observation for another week revealed no further differentiation of eyespots in these worms.

Experiments with sodium ribonucleate, one-day exposures

In this series of experiments 45 planaria were starved for 14 days, the heads and tails extirpated and the remainders of these worms were cut into six pieces of equal size (Fig. 1). Fifteen sections from each level were used in each of three test solutions: (1) 0.01% sodium ribonucleate, (2) 0.005% sodium ribonucleate, and (3) boiled tap water. The worms were exposed to sodium RNA solutions for

TABLE II

Regeneration of eyespots after a one-day exposure to yeast sodium ribonucleate; worms starved for 14 days

Medium	Level of section	Total number of sections	Number with eyespots					
			4	5	6	7	8	9
			Days after cutting					
A. Sodium RNA (0.01%)	1	12	5	7	10	11	11	11
B. Sodium RNA (0.005%)	1	14	8	11	13	13	13	13
C. Tap water	1	14	4	6	11	13	13	13
A.	2	14	—	1	1	2	3	3
B.	2	14	—	2	2	2	3	3
C.	2	14	—	1	2	5	6	6
A.	3	12	—	1	1	1	1	1
B.	3	14	—	2	2	2	3	3
C.	3	13	—	—	1	1	1	1
A.	4	15	—	—	—	—	1	1
B.	4	15	—	1	1	1	1	2
C.	4	14	—	—	—	—	—	—
A.	5	13	—	2	3	3	3	4
B.	5	14	—	1	1	1	1	1
C.	5	14	—	—	1	1	1	1
A.	6	15	—	2	8	12	12	12
B.	6	13	—	6	9	11	11	12
C.	6	14	—	2	5	6	6	6

Table III

Regeneration of eyespots after a one-day exposure to yeast sodium ribonucleate; worms starved for five days

Medium	Level of worm	Total number of sectioned pieces	Number with eyespots						
			4	5	6	7	8	9	10
			Days after cutting						
A. Sodium RNA (0.01%)	1	14	1	4	5	6	6	7	9
B. Sodium RNA (0.005%)	1	15	1	3	6	8	10	11	11
C. Tap water	1	15	—	3	6	8	9	10	11
A.	2	14	—	—	—	1	2	2	2
B.	2	14	—	—	—	2	3	4	4
C.	2	15	—	—	—	—	—	1	2
A.	5	15	—	2	2	2	2	2	2
B.	5	15	—	—	—	—	—	—	—
C.	5	15	—	—	—	1	1	1	1
A.	6	15	—	3	9	11	11	11	11
B.	6	15	—	7	13	13	13	13	13
C.	6	15	—	3	5	7	9	9	10

the 24-hour period after cutting, and then transferred to boiled tap water. Regeneration was followed up to 20 days after cutting, but differentiation was complete 9 days after cutting. The results are shown in Table II. In the most posterior sections of the worms (level 6), both concentrations of sodium RNA seemed to have stimulated a higher incidence of eyespot differentiation seven days after sectioning the worms.

This experiment was repeated in the same way with a group of worms that had been starved for five days, and stimulation of time of eyespot formation occurred at the posterior level. These results are presented in Table III. The results of cut sections 3 and 4 are omitted in the table because these regions regenerate poorly, presumably due to the mechanical interference of the pharynx. The effects of RNA in speeding the time of differentiation of the eyespots are evident at level 6, and this effect is demonstrated to a slight extent at level 2.

Experiments with Horse Serum, Amino Acids, Chick Embryo Extract, Sodium Ribonucleate and Tap Water: Continual Exposure

From worms starved for 30 days, 10 cut sections from each of the four regions 1, 2, 5 and 6 (Fig. 1) were cultured in each of the following separate solutions: 5% horse serum, 0.5% essential plus 0.5% non-essential amino acids of Eagle's medium, 1% chick embryo extract, 0.005% yeast sodium ribonucleate and boiled tap water. These media were diluted to the test concentrations by addition of Seitz-filtered tap water which contained a 1% penicillin-streptomycin mixture. The results are given in Table IV. It is evident that the normal gradient of head fre-

Table IV

Regeneration of eyespots upon continuous exposure to various nutrient solutions and tap water; worms starved for 30 days

Medium	Level of worm	Total number of sectioned pieces	Number with eyespots								
			4	5	6	7	8	9	10	11	12
			Days after cutting								
H.S.—Horse serum (5%)	1	9	1	3	5	7	7	7	7	8	8
A.A.—Eagle's essential and non-essential amino acids (0.5% each)	1	7	1	2	4	4	5	5	5	5	6
E.E.—Chick embryo extract (1%)	1	7	1	3	5	7	7	7	7	7	7
RNA—Yeast sodium ribonucleate (0.005%)	1	7	—	3	6	7	7	7	7	7	7
H_2O—Boiled tape water		8	—	—	2	3	3	5	5	6	7
H.S.	2	8	—	—	2	2	2	2	3	3	3
A.A.	2	8	—	1	2	3	5	5	6	6	6
E.E.	2	5	—	—	1	1	2	3	3	4	4
RNA	2	10	—	1	2	2	4	4	5	6	6
H_2O	2	8	—	—	1	2	2	3	4	5	6
H.S.	5	8	—	—	2	2	2	2	2	2	2
A.A.	5	6	—	1	3	3	4	4	4	4	4
E.E.	5	10	—	—	1	1	1	1	1	1	1
RNA	5	9	—	—	1	1	1	1	1	1	1
H_2O	5	10	—	—	1	1	3	3	3	3	4
H.S.	6	8	1	3	5	5	7	7	7	7	7
A.A.	6	8	—	2	4	4	6	6	6	6	6
E.E.	6	8	—	2	4	4	4	6	7	8	8
RNA	6	10	—	1	3	3	4	4	5	6	8
H_2O	6	10	—	—	2	5	5	5	6	6	6

quency that is observed in worms regenerating in tap water is modified in sections 1 and 6 by each of the test solutions, as shown by acceleration of the time of eyespot differentiation. In these experiments the extent of eyespot regeneration did not seem to vary between the nutrient solutions and tap water controls at the various levels.

Isotopic Experiments with Worms Exposed to Nutrient Medium

In order to test the assumption that the nutrient medium had stimulated protein synthesis, studies using labeled CO_2 were undertaken. Worms starved for three weeks were divided into two groups of 60. Group I was cut to provide three regenerating pieces, each with two wound surfaces, per intact worm (1 + 2, 3 + 4, 5 + 6; Fig. 1). Group II had only the heads and tails removed to provide one regenerating piece, each with two wound surfaces, per intact worm. Each group was divided in half, 30 pieces of each kind being cultured in nutrient medium and 30 in Seitz-filtered tap water. The nutrient medium was the same as previously used, but horse serum was omitted and exposure lasted four days. At the end of this period the regenerating pieces were exhaustively washed over a four-hour period and then incubated separately, each with 150 μc. $C^{14}O_2$, for three hours, according to the method of Flickinger (1959). The incubation was terminated by the addition of cold 10% TCA. The regenerating pieces of Group II were cut in three pieces, pre-pharyngeal, pharyngeal and post-pharyngeal, which corresponded to the three levels of Group I. Following homogenization and washing with 10% TCA, the residue was treated with hot 5% TCA to hydrolyze nucleic acids, and then lipids were extracted. The residue, designated as the protein fraction, was dissolved in 0.5 ml. 0.2 N NH_4OH, plated on tared planchets, weighed, and counted in a gas-flow counter. Self-absorption was not a significant source of error. The results of this experiment (Table V) clearly demonstrate that stimulation of protein synthesis discernible by $C^{14}O_2$ incorporation is greatest in the posterior levels. It is noteworthy that the axial gradient of incorporation (Flickinger, 1959) is preserved in the tap water controls, but markedly altered in those from nutrient medium.

TABLE V

Effect of nutrient medium on stimulation of $C^{14}O_2$ incorporation into protein of regenerating worms; worms starved three weeks

Medium	Level of section*	Group I		Group II	
		CPM/MG Protein	% Stimulation	CPM/MG Protein	% Stimulation
Tap water	1 + 2	8,928	—	9,672	—
	3 + 4	7,896	—	9,139	—
	5 + 6	6,169	—	6,502	—
Nutrient medium**	1 + 2	2,776	−322	15,605	+161
	3 + 4	9,947	+126	6,976	−131
	5 + 6	11,545	+187	19,770	+304

* See Figure 1.

** The nutrient medium was the same as in Table I except for the omission of horse serum.

DISCUSSION

The results of this investigation clearly support previous work (Child, 1941) and demonstrate that an anterior-posterior gradient of head frequency exists for the time of appearance and completeness of development of eyespots in pieces of regenerating planaria. Furthermore, the data indicate that if sodium ribonucleate, horse serum, amino acids, or chick embryo extract are added to the medium, the number of eyespots forming and the rate of their appearance are usually increased, particularly at the posterior levels of the worm. This effect is more striking in worms that have been starved for longer periods, but even in starved worms the extent of eyespot formation is sometimes not affected, although the rate of eyespot regeneration is stimulated. Regeneration is usually quite poor in pieces in which the pharynx occupies the majority of the piece; thus such regions were not utilized in some of the experiments.

Some attempts were made to reverse the polarity of regenerating pieces by immersing the prospective posterior end of the worms in 0.005% sodium ribonucleate in the agar-slab type of experiment (Flickinger, 1959), but these experiments were unsuccessful. The compounds used in this study, which we have designated as nutrients, can accelerate differentiation of cephalic structures, an effect which is more pronounced at posterior levels. Despite the fact that these nutrients cannot be demonstrated to affect polarity in the agar-slab experiments, as chloramphenicol does, they do stimulate the rate and extent of cephalic regeneration, suggesting that their availability influences the rate of protein synthesis. Previous mention has been made of the evidence relating a gradient of protein synthesis to the biological polarity in the regenerating planarian (Flickinger, 1959; Flickinger and Coward, 1962).

The results of our earlier experiments show that this stimulation is greater at posterior levels of the worms where isotopic data show a lower rate of incorporation of CO_2 and glycine into protein (Flickinger, 1959). The present isotopic data demonstrate that nutrients stimulate protein synthesis asymmetrically in respect to the normal axial gradient. This is in harmony with the observed biological effect—the increase of head frequency at posterior levels. The fact that substrates and cofactors necessary for protein synthesis can stimulate the rate of eyespot formation in cut posterior regions of the worms more than at the anterior end suggests that these materials are not present at optimal levels in the posterior parts of the worms. This contention is in agreement with the hypothesis that head formation occurs at the cut end of the worm with the highest level of activity of the protein-synthesizing mechanisms. Furthermore, the inhibitory role of the head in preventing posterior cephalic differentiation may be due to its capacity to drain substrates and cofactors from the more posterior levels.

SUMMARY

1. Planaria, *Dugesia dorotocephala,* were starved for periods of 5–30 days, the heads and tails removed and the remainders of the worms were cut into three, four or six pieces. These sections were allowed to regenerate in tap water or in solutions containing yeast sodium ribonucleate (0.005–0.01%), horse serum (5%), amino acids (1%), and chick embryo extract (1%). The media were changed every second day. The cultures were observed daily and the time of eyespot formation was recorded.

2. All of the nutrient solutions tested were effective in promoting the rate of regeneration of eyespots, particularly in the posterior pieces in which eyespots appeared 1–2 days sooner than in the tap water controls. The effect of the test solutions upon the extent of eyespot regeneration is less consistent, but some stimulation of differentiation at the posterior levels was observed.

3. Exposure to yeast sodium ribonucleate for only a day after cutting the worms also stimulated head frequency posteriorly, but this is not as effective as continual exposure.

4. The biological results were confirmed by $C^{14}O_2$ incorporation studies which demonstrated increased synthetic activity in posterior levels of the worms that had been exposed to the nutrient medium.

LITERATURE CITED

Brønsted, A., and H. V. Brønsted, 1953. The acceleration of regeneration in starved planarians by ribonucleic acid. *J. Embryol. Exp. Morph.*, **1**: 49–54.

Child, C. M., 1941. Patterns and Problems of Development. University of Chicago Press, Chicago.

Flickinger, R. A., 1959. A gradient of protein synthesis in planaria and reversal of axial polarity of regenerates. *Growth*, **23**: 251–271.

Flickinger, R. A., and S. J. Coward, 1962. The induction of cephalic differentiation in regenerating *Dugesia dorotocephala* in the presence of the normal head and in unwounded tails. *Develop. Biol.*, **5**: 179–204.

Niu, M. C., and V. C. Twitty, 1953. The differentiation of gastrula ectoderm in medium conditioned by axial mesoderm. *Proc. Nat. Acad Sci.*, **39**: 985–989.

OSMOREGULATORY ROLE OF THE ANTENNARY GLAND IN TWO SPECIES OF ESTUARINE CRABS[1]

PAUL A. DEHNEL AND DMITRY STONE[2]

Department of Zoology, University of British Columbia, Vancouver 8, British Columbia, Canada

Osmotic regulation in aquatic animals has been reviewed by Krogh (1939), Beadle (1957) and discussed more recently by Prosser and Brown (1961) and Lockwood (1962). Beadle (1957, p. 335) has commented on the evolution of osmotic regulation in Crustacea, and postulated "that in marine crabs there are at least two sets of active processes at work, in the gills and in the excretory organs, which are responsible for the ionic imbalance between blood and sea water." He suggested that adjustments in the rates of these processes could have led, in appropriate environments, to the evolution of both hypo- and hyper-osmotic regulation. Osmotic behavior of nine species of eastern Pacific crabs was investigated by Jones (1941), who categorized *Hemigrapsus oregonensis* and *H. nudus* as hyper-osmotic regulators, without any capacity for hypo-osmotic regulation. Gross (1961) recently reported *Hemigrapsus oregonensis* to be a strong hypo-osmotic regulator in sea water as high as 175%. The mechanisms by which crabs establish and maintain osmotic and ionic gradients between their internal and external environments have been studied (Nagel, 1934; Green, Harsch, Barr and Prosser, 1959). In *Carcinus maenas,* a crab showing no hypo-osmotic regulation, Prosser and Brown (1961, p. 14) have suggested three mechanisms which play a part in hyper-osmotic regulation: "low permeability to water and salts, increased fluid output, particularly of urine, and active salt absorption from the medium." Participation of the antennary glands in hyper-osmotic regulation in *Pachygrapsus crassipes,* a species which regulates in both low and high salinities, has been suggested (Prosser, Green and Chow, 1955). Gross (1957a) has submitted that active absorption of water may be a method of hypo-osmotic regulation. The antennary glands have been considered to be more important in ionic than total osmotic regulation (Prosser, Green and Chow, 1955; Green, Harsch, Barr and Prosser, 1959; Prosser and Brown, 1961). Evidence for this viewpoint has been obtained mainly from the work of Nagel (1934), Webb (1940), Robertson (1949) and Parry (1954). In a semi-terrestrial crab, *Coenobita perlatus,* Gross and Holland (1960) demonstrated behavioral mechanisms for regulation of osmotic concentration of the blood. The antennary glands in this species were shown to contribute only to the regulation of potassium and not total osmotic regulation. The ratio, urine concentration/blood concentration (U/B ratio), for specific ions in selected regulating and adjusting

---

[1] This study was aided by grants from the National Research Council of Canada and the National Science Foundation of the United States.

[2] Present address: Department of Mines and Natural Resources, Fisheries Branch, Province of Manitoba, Winnipeg 1, Manitoba.

Crustacea, has indicated that the antennary glands do act selectively to control certain ionic imbalances between blood and external media (Peters, 1935; Picken, 1936; Prosser, Green and Chow, 1955; Gross, 1959; Gross and Holland, 1960; Prosser and Brown, 1961).

The effects of temperature on osmotic regulation in various aquatic organisms have been investigated (Wikgren, 1953; Dehnel, 1962) and reviewed, in part, by Verwey (1957). The effect of external salinity on animal activity, particularly on osmotic behavior and water and ion fluxes between body fluids and media, and concomitant weight changes have been studied (Jones, 1941; Robertson, 1949, 1953; Gross, 1954, 1955, 1957a; Prosser, Green and Chow, 1955; Dehnel, 1960, 1962).

The osmotic behavior of *H. oregonensis* and *H. nudus* has been studied with respect to blood responses over a range of experimental temperatures and salinities (Dehnel, 1962). These two species are established in this geographical area with seasonal temperature and salinity cycles similar to those discussed by Broekema (1941) and Verwey (1957). The activity of the antennary glands in *Hemigrapsus* demonstrates that the osmoregulatory responses of these species change significantly with seasonal field temperature and salinity.

MATERIAL AND METHODS

Two species of shore crabs, *Hemigrapsus nudus* (Dana) and *H. oregonensis* (Dana), were collected at two seasons, summer and winter, from the intertidal zone at Spanish Bank, Vancouver, British Columbia.

In the laboratory the animals were divided into four groups, and depending on size, experimental salinity and temperature, the number per plastic tray varied from 10 to 15 animals. Each group provided three sequential sets of 10 separate urine samples.

To bring the animals to a common osmotic level each group was totally immersed for 36 to 48 hours in 75% sea water, a suitable intermediate salinity for equilibration. Trays were placed in darkened refrigerators set at the experimental temperature. Following equilibration, each group was transferred to 4.0 liters of water at experimental temperature and salinity conditions. Animals were not fed and sea water was renewed daily.

Experimental temperatures, summer and winter, were 5°, 15°, and 25° C. (±1.0° C.), and experimental salinities were 6%, 12%, 25%, 75%, 100%, 125%, 150% and 175% sea water, based on a standard sea water: 31.88‰ salinity and 17.65‰ chlorinity at 25° C. Experimental salinities were obtained either by diluting sea water with distilled water or by concentrating it with appropriate amounts of sea salt. Salinities were determined by a 1000-cycle conductivity bridge calibrated to the standard sea water, and alternatively by titration. Field and experimental salinities are expressed as percentage sea water based on the above standard.

After 3, 24 and 48 hours at the experimental conditions, urine was sampled by means of glass capillary tubes, 0.40 mm. inside diameter and 1½ inches long, drawn to a fine tip and inserted into a small rubber pipette bulb. Crabs were blotted dry and manipulated under a binocular microscope, so that the tip of a blunt needle, mounted on the microscope stage, could be inserted under the operculum, which covers the pore. As the operculum was raised, the tip of the capillary tube was inserted beneath it. This usually resulted in a discharge of urine. If necessary, gentle pressure, exerted dorso-ventrally on the body of the crab, would cause expulsion of urine. The tubes were sealed immediately with "Seal-Ease" and quick-frozen on dry ice. Samples were then transferred to a brine solution at −15° C., until needed. The animals were returned to the ex-

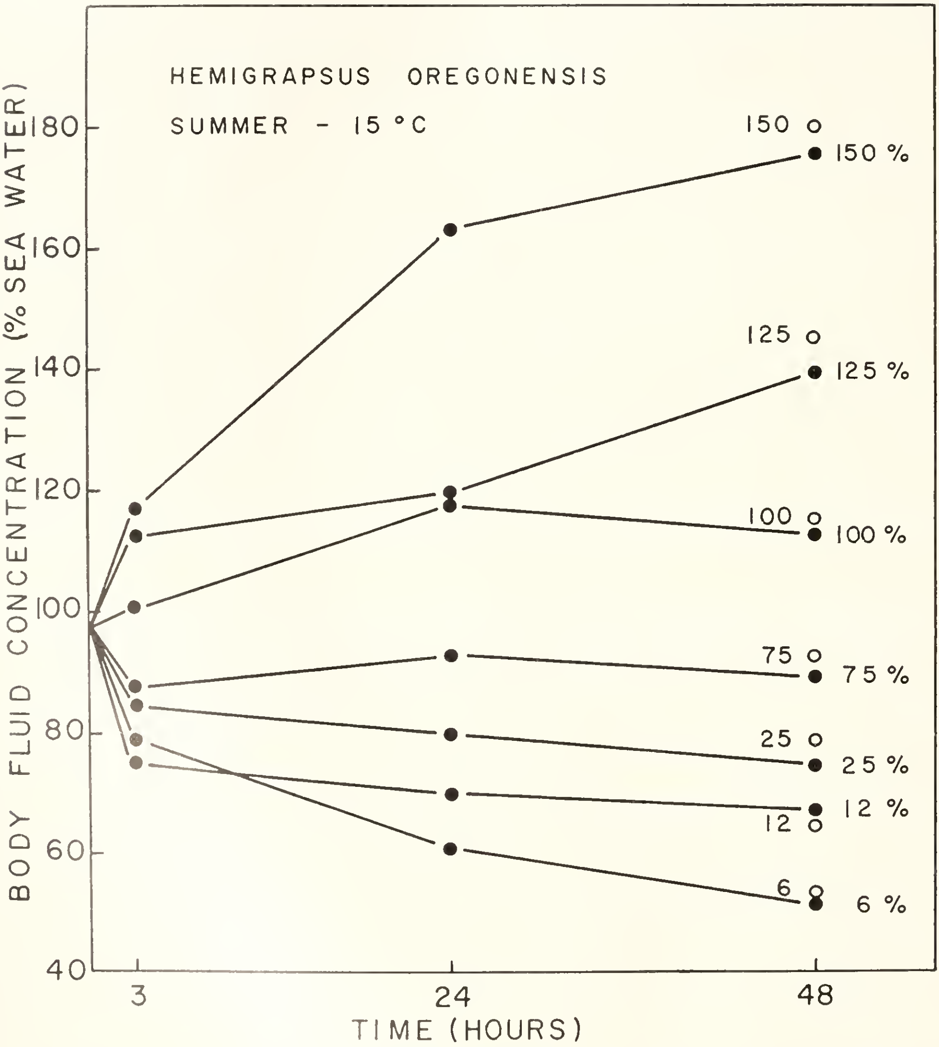


FIGURE 1. Urine concentration changes and 48 hour blood values in summer *Hemigrapsus oregonensis*, at 15° C. as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 animals. Urine values are indicated by closed circles (●), blood values (Dehnel, 1962), by open circles (○).

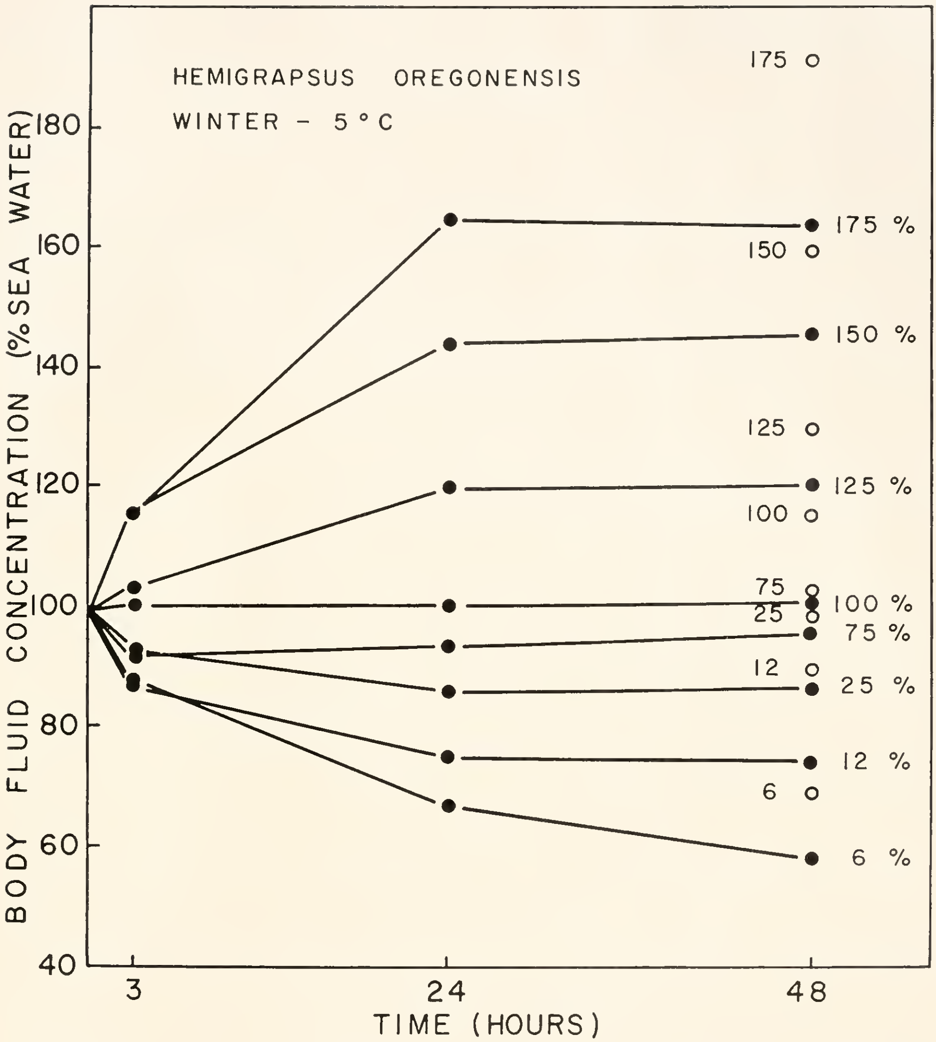


FIGURE 2. Urine concentration changes and 48-hour blood values in winter *Hemigrapsus oregonensis,* at 5° C., as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 animals for each time period. Urine values are indicated by closed circles (●), blood values (Dehnel, 1962), by open circles (○).

perimental conditions after the 3-hour and 24-hour samplings, and discarded after 48 hours. Measurement of the total osmotic concentration of urine samples was accomplished by the method of melting point determination described by Jones (1941) and modified by Gross (1954).

The data were analyzed for salinity and temperature effects and seasonal differences by means of Student's "t" test. The same test was applied to the differences between mean osmotic concentrations of blood (Dehnel, 1962) and urine from similarly treated animals. Unless otherwise stated, statistical significance is attributed to P values <0.01.

RESULTS

Response to external salinity changes

Osmotic responses of *H. oregonensis* and *H. nudus* to a range of experimental salinities are shown in Figures 1 to 4. Forty-eight-hour blood concentration values (Dehnel, 1962) are included for comparison. In each figure, urine concentration at time zero is an arbitrary mean of all three-hour values used in the figure. The 3-, 24- and 48-hour values for animals in 75% sea water were obtained after 36 to 48 hours' equilibration in that medium. Responses common to the four sets of curves are the rise in urine concentration with time in high salinities, and the fall in low salinities, at rates directly related to the gradients between media and urine concentrations at time zero.

Hemigrapsus oregonensis

Summer animals (Fig. 1) did not survive 48 hours in 175% sea water. In general, changes in blood and urine concentrations were rapid, and a steady-state

TABLE I

Comparison of 48-hour urine and blood concentrations (urine/blood ratio and urine minus blood gradient) for summer-adapted and winter-adapted animals at three experimental temperatures, 5°, 15° and 25° C., and three experimental salinities, 12%, 75% and 125% sea water. P values apply to differences between concentration of blood and urine

Exp. sal. (% S.W.)	(° C.)	12%			75%			125%		
		U/B	U-B	*P* value	U/B	U-B	*P* value	U/B	U-B	*P* value
					Summer					
H. nudus	5	0.84	−11.7	<0.010	0.97	−2.4	N.S.	0.94	−8.3	N.S.
H. oreg.	5	0.90	−6.2	N.S.	0.99	−1.3	N.S.	—	—	—
H. nudus	15	1.01	0.9	N.S.	0.99	−0.6	N.S.	0.98	3.3	N.S.
H. oreg.	15	1.02	1.3	N.S.	0.95	−4.6	<0.001	0.96	−5.7	<0.01
H. nudus	25	0.98	−1.0	N.S.	0.98	−1.7	N.S.	0.98	−2.4	N.S.
H. oreg.	25	0.98	−1.1	N.S.	1.04	3.3	N.S.	1.00	−0.2	N.S.
					Winter					
H. nudus	5	0.75	−19.3	<0.001	0.83	−19.8	<0.001	0.83	−24.3	<0.001
H. oreg.	5	0.83	−15.1	<0.001	0.95	−5.0	N.S.	0.93	−9.4	<0.005
H. nudus	15	0.67	21.9	<0.001	0.97	−2.5	N.S.	0.77	−33.6	<0.001
H. oreg.	15	0.63	31.1	<0.001	0.77	−22.9	<0.001	0.86	−17.7	<0.001
H. nudus	25	0.63	28.3*	—	0.87	−12.7*	—	0.82	−24.4	<0.001
H. oreg.	25	0.69	−25.1*	—	0.87	−12.1	<0.001	0.88	−15.4	<0.001

* = 24-hour blood values.
N.S. = not significant.

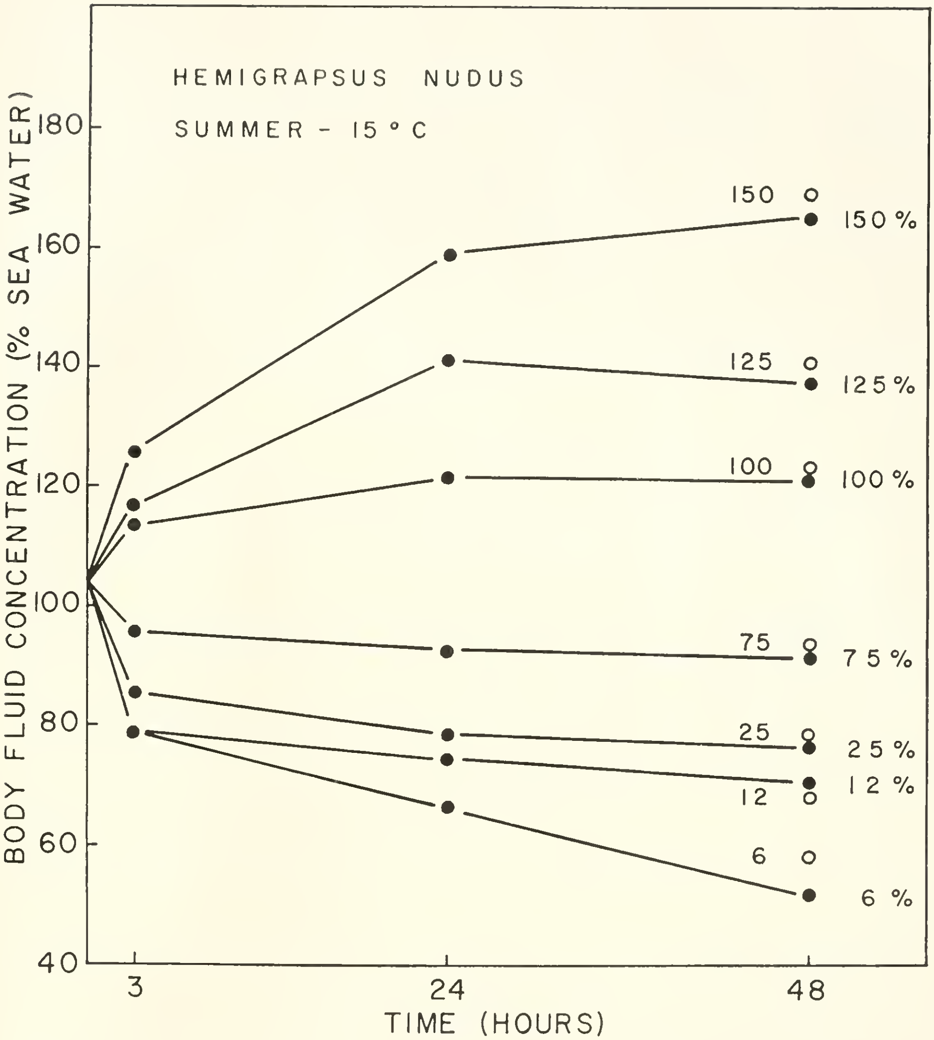


FIGURE 3. Urine concentration changes and 48-hour blood values in summer *Hemigrapsus nudus,* at 15° C., as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 animals for each time period. Urine values are indicated by closed circles (●), blood values (Dehnel, 1962), by open circles (○).

was reached after 24 hours' exposure, with few exceptions. It was assumed, however, that all major changes in the concentrations of both body fluids had occurred at the end of 48 hours. Urine concentrations were hyper-osmotic to high salinities. Blood and urine was isosmotic, except at 75% and 125% sea water, where the blood was significantly higher (Table I). Hyper-osmoticity of the urine in high salinities was probably the result of continued absorption of salts by gill and gut

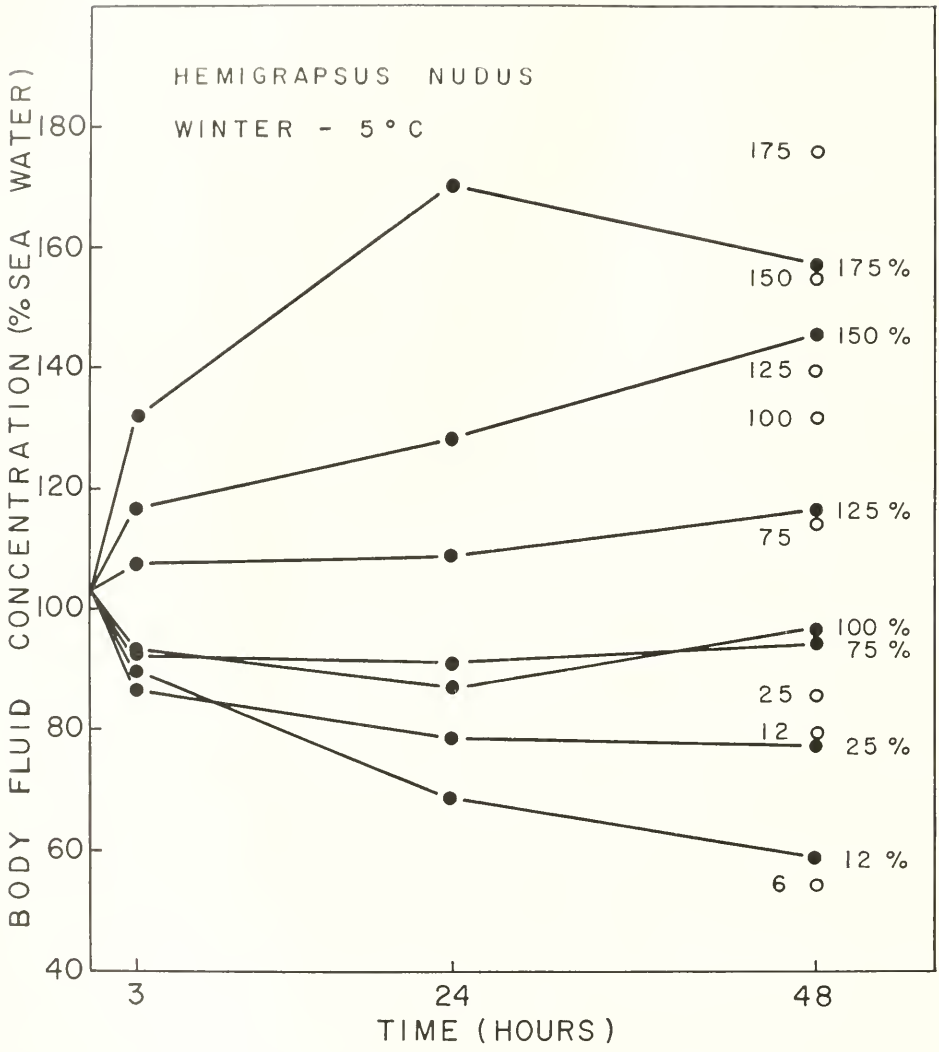


FIGURE 4. Urine concentration changes and 48-hour blood values in winter *Hemigrapsus nudus*, at 5° C., as a function of time in the experimental salinities. Each point represents the mean of the measurements of 10 animals for each time period. Urine values are indicated by closed circles (●), blood values (Dehnel, 1962), by open circles (○).

tissues and a concomitant loss of water to the external media through the integument. In salinities below 100% sea water hyper-osmotic regulation occurred.

Winter animals (Fig. 2) survived 48 hours in 175% sea water, probably reflecting adaptation to higher salinity field conditions. Again, the blood and urine curves reached equilibration after 24 hours. In high salinities, urine was hypo-osmotic to both the blood and the media. As in summer animals, at salinities below 100%, hyper-osmotic regulation occurred.

Hemigrapsus nudus

Urine and blood concentration changes occurred in this species at the same rate as in *H. oregonensis*. Summer animals (Fig. 3) survived in 150% but not in 175% sea water. Blood and urine were isosmotic (Table I), and both fluids were hyper-osmotic to all experimental salinities. In winter animals (Fig. 4) the separation of the urine curves, especially in 75% and 100% sea water, was not as clear as in the summer experiments. Animals survived 48 hours in 175%. The lower survival limit of animals from which urine was collected was 12%, and the 48-hour concentration was significantly lower than the comparable summer value. Urine concentrations were hypo-osmotic to the media in high salinities, whereas blood concentrations were hyper-osmotic, but approached isosmoticity at 150% and 175% sea water. Over the entire salinity range blood concentrations were significantly higher than urine.

Interspecific comparison

The absolute difference between 48-hour urine concentrations for *H. oregonensis* in 6% and 150% sea water was 125% in summer and 88% in winter. For *H. nudus*, the difference in 12% and 150% sea water was 92% in summer and 86% in winter. In both species higher summer urine concentrations in media above 100% sea water accounted for this difference. A similar comparison for blood resulted in an identical difference for *H. oregonensis*, summer and winter, and for *H. nudus* the differences were approximately the same (100% in summer, 76% in winter).

In summer, blood and urine concentrations for *H. nudus* showed a more constant response than *H. oregonensis* in the high salinities. This suggests that *H. nudus* potentially is the better regulator in high salinities. In low salinities, the abilities of both species to hyper-osmoregulate were similar. In winter, *H. oregonensis* showed the more constant response to high salinities, and regulated to a greater degree in salinities lower than 100%.

Seasonal effect of salinity

The abilities of the two species to establish and maintain osmotic gradients between their body fluids and the external media changed from summer to winter. To evaluate seasonal effects, gradients were derived from the data of Figures 1 to 4 and from Dehnel (1962).

Hemigrapsus oregonensis

When the gradient between blood concentration and experimental sea water is compared, winter crabs maintained a significantly greater gradient below 100% sea water; above 100%, summer crabs maintained the greater gradient (Fig. 5). Winter urine was hyper-osmotic to summer urine in low salinities, and above 100% sea water, hypo-osmotic to summer urine and the media.

Comparison of the seasonal gradients between blood and urine concentrations is given in Figure 6. At all salinities except 75% the winter gradient is significantly higher. This is mainly accounted for by the fact that winter blood maintains a proportionately greater gradient relative to the experimental salinities, and to the urine, whereas summer blood and urine are essentially isosmotic. Winter crabs would appear to be better regulators in hypo-osmotic media, and summer crabs, in hyper-osmotic media (Fig. 5). Further, the isosmotic condition be-

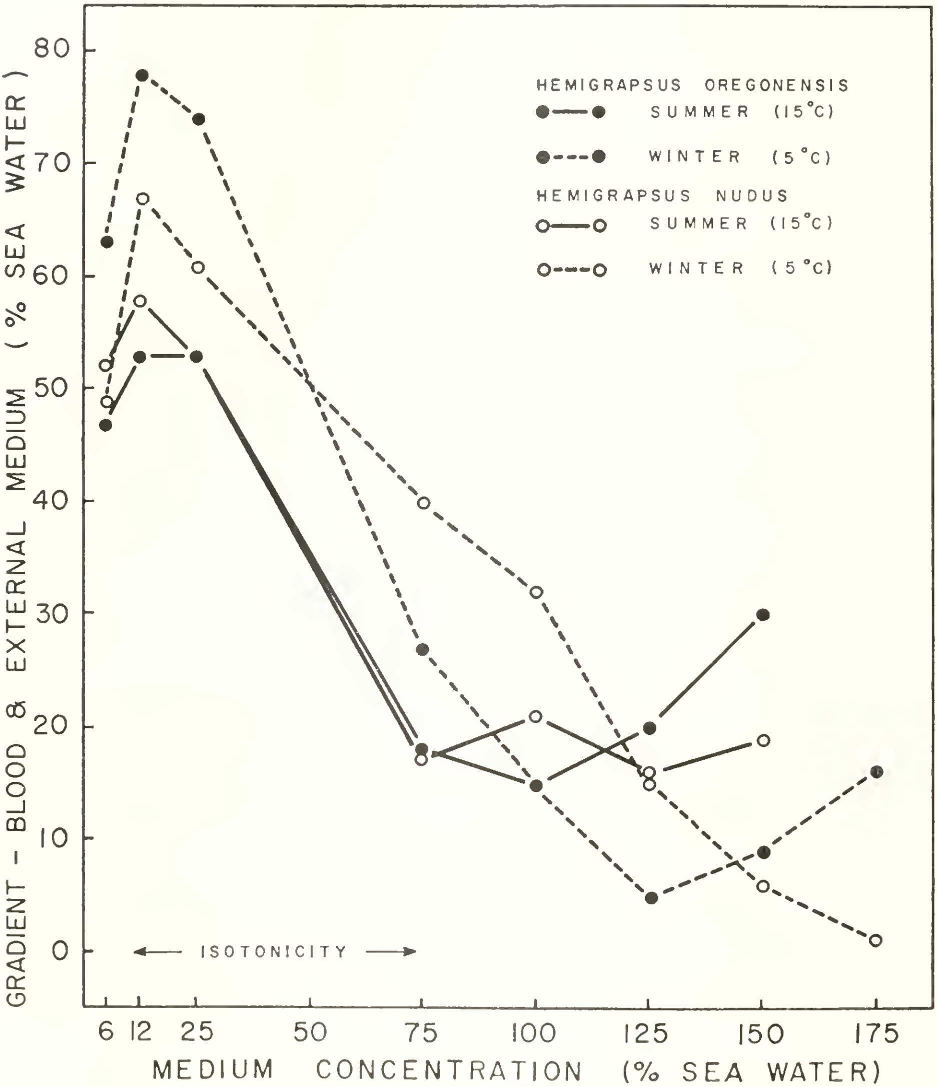


FIGURE 5. Relationship of the gradient between blood and external sea water to medium concentrations in summer (15° C.) and winter (5° C.) *Hemigrapsus oregonensis* and *Hemigrapsus nudus*, after exposure for 48 hours to the experimental salinities. For the purposes of comparison, blood data have been inserted from a previous publication in this journal (Dehnel, 1962).

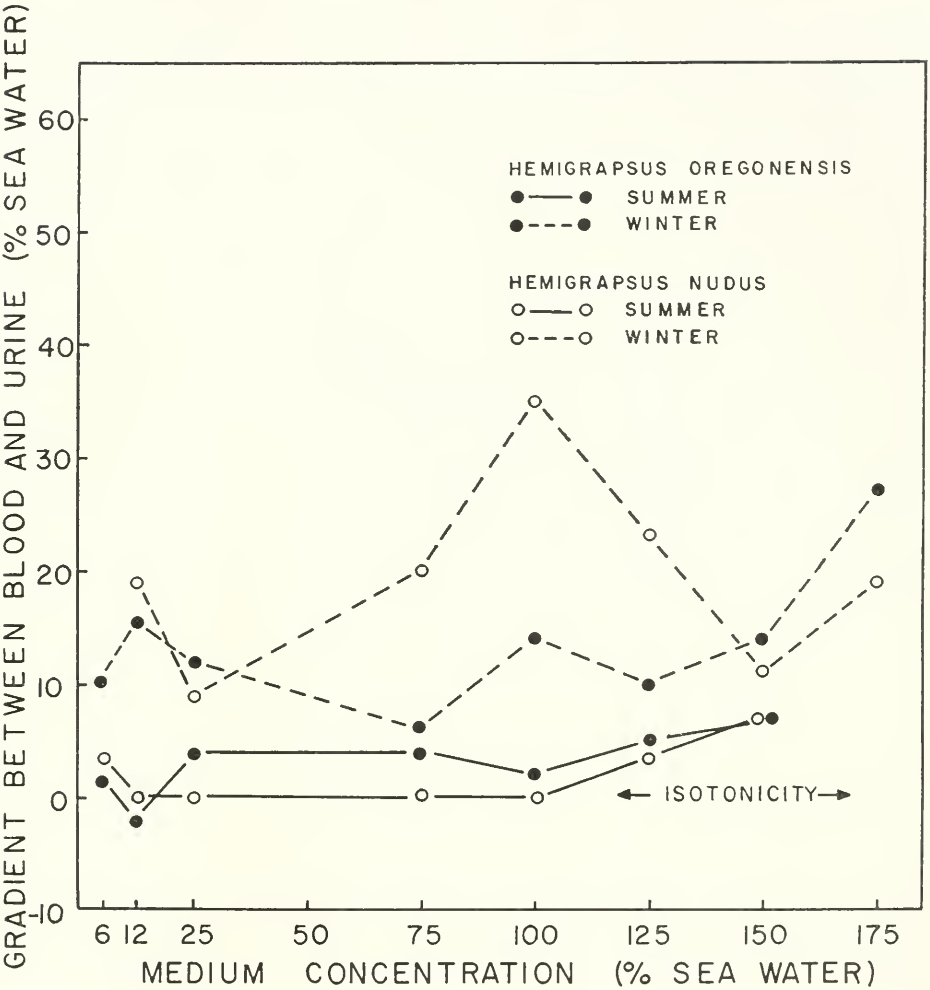


FIGURE 6. Relationship of the gradient between blood and urine to medium concentrations in summer (15° C.) and winter (5° C.) *Hemigrapsus oregonensis* and *Hemigrapsus nudus*, after exposure for 48 hours to the experimental salinities. Blood values are from Dehnel (1962).

tween summer blood and urine implicates extra-renal mechanisms in hyper-osmotic regulation. Production of hypo-osmotic urine by winter-adapted animals suggests the participation of the antennary glands in hyper-osmotic regulation.

Hemigrapsus nudus

The results for *H. nudus* in general paralleled those for *H. oregonensis*. The blood concentration gradient for winter crabs was the greater below 125% sea water, and above this, summer crabs maintained the greater gradient (Fig. 5). As salinity increased, winter crabs steadily decreased their gradient, and attained isosmoticity at 175% sea water. Summer and winter urine from 25% to 75% sea water was isosmotic. At higher salinities summer urine was significantly hyper-osmotic to winter urine and to the media. Winter urine was isosmotic at 100% and significantly hypo-osmotic at higher salinities.

Blood and urine comparison (Fig. 6) shows that at all salinities the winter gradient was significantly higher. Again, the higher winter gradient can be accounted for by the fact that summer blood and urine are isosmotic. From these data it would appear that winter crabs are the better regulators.

Interspecific comparison

Winter blood concentration gradients of both species were higher than corresponding summer ones at salinities below approximately 100% sea water. Above that salinity summer crabs had higher gradients and winter crabs approached isosmoticity (Fig. 5). Urine concentration gradients in general were similar to those of the blood, except that absolute differences were less in low salinities and greater in high salinities. Blood-urine differences for winter crabs were, in general, much greater for *H. nudus*, whereas summer differences were much less, and *H. oregonensis* tended to maintain the greater difference between blood and urine (Fig. 6).

TABLE II

Seasonal comparison for both species of 48-hour urine (upper values) and blood (lower values) concentrations of summer-adapted animals (S) and winter-adapted animals (W) at 5°, 15° and 25° C., measured at a series of experimental salinities

Experimental salinities	(°C.)	Body fluid concentration (% S.W.)											
		12%		25%		75%		100%		125%		150%	
		S	W	S	W	S	W	S	W	S	W	S	W
H. nudus	5	64 75	59* 79*	71 85	78* 86*	91 93	95* 115	123 116	97 132	138 146	117 140	155 173	146* 156
H. oreg.	5	58 64	74 89	78 73	87 99	95 96	96* 105	121 117	101 115*	138 144	120 130	— —	— 159
H. nudus	15	71 70	45 66	76 78	84* 83*	92 92	94* 96*	121 121	91 114	138 141	111 144*	— 167	— 159
H. oreg.	15	67 65	54* 85	74 78	73* 88	88 93	77 100	112 115	91 111*	139 145	112 130	— 180	— 158
H. nudus	25	60 61	18	76 76	71* 79*	89 90	81* 97	123 115	97 112*	138 140	112 133	— —	— 156
H. oreg.	25	71 69	57 82	76 73	76* 82	93 90	78 90*	109 108	88 113*	135 135	112 127	174 —	138 168

* = not significant.

Effect of temperature

Table II compares 48-hour blood and urine concentrations at 5°, 15° and 25° C. for summer and winter animals over a salinity range from 12% to 150% sea water. Blood and urine changes for seasonally adapted crabs have been discussed in the previous section. These data represent, in part, an effect of temperature which is due to seasonal change.

Hemigrapsus oregonensis

At 5° C., below 75%, winter animals showed significantly higher urine concentrations than summer animals. Above this salinity, summer values exceeded winter ones. At 15° C. no difference was noted in low salinities, but at higher salinities, summer values were significantly higher than their winter counterparts. At 25° C. summer values significantly exceeded winter values in all salinities except 25% in which they were the same.

Blood concentrations were hyper-osmotic to all experimental salinities at all temperatures, summer and winter. At salinities less than 100%, blood concentrations of winter animals at 5° and 15° C. were higher than those of summer animals at any of the temperatures. Above this salinity, blood of summer animals at the two lower temperatures was significantly higher when compared with winter crabs. At lower salinities the highest blood concentration, for winter animals in particular, was found generally at the lowest temperature. As the concentration of the experimental salinity decreased, blood concentration increased as temperature decreased.

Hemigrapsus nudus

At 5° C., in salinities of 75% and less, no significant differences were observed between summer and winter urine concentrations. At higher salinities, summer values significantly exceeded winter ones. At 15° and 25° C. summer and winter urine concentrations were similar, in 25% and 75% sea water, but in both lower and higher salinities, summer values were significantly higher.

Blood concentrations were hyper-osmotic to all experimental salinities at all temperatures, summer and winter. Over the salinity range, 6% to 125%, blood concentrations for summer or winter crabs were similar, except for the 5° C. winter data. At the lower salinities, both summer and winter groups, the highest blood concentration generally was found at the lowest temperature, even though the temperature effect on blood concentration at a given salinity was relatively slight for summer animals.

Interspecific comparison

Urine data suggest that *H. oregonensis* shows seasonal differences in concentration, and temperature effect during a given season, over a wider range of conditions than *H. nudus*. The principal differences in response between these two species occurred in salinities between 12% and 75%. Comparison of summer blood of the two species shows this body fluid to be similar over most of the salinity range. Major differences occur only at the higher salinities, where *H. oregonensis* is the higher. *H. nudus* has the higher winter blood concentration over the major portion of the salinity range. At the lower salinities *H. oregonensis* maintains a higher blood gradient.

Urine-blood ratio (U/B)

Summer U/B ratios approached unity in both species (Table I). In most of the selected conditions, blood was more concentrated than urine. Where U/B ratios >1.0, the departure from unity was not significant. Blood was significantly hyper-osmotic to urine in *H. oregonensis* in 75% and 125% sea water at 15° C., and in *H. nudus*, only in 12% at 5° C.

Winter U/B ratios in both species in selected conditions were all lower than comparable summer ones. The absolute differences in concentration between urine and blood increased to statistically significant levels in most of these conditions. The increases were due to a generally larger net decrease in urine concentration than blood from summer to winter in similar experimental conditions. Blood values were significantly higher than urine values in all conditions, except for two shown in Table I.

At 5° C., *H. oregonensis*, winter and summer, had higher U/B ratios than *H. nudus* over the entire range of salinities. In winter at 15° and 25° C., and in summer at 25° C for the same salinities, *H. nudus* ratios were higher, but the differences were significant only in winter animals at 25° C.

DISCUSSION

Hyper-osmotic regulation of blood concentration in *H. oregonensis* and *H. nudus* was demonstrated by Jones (1941). Gross (1957a) showed some degree of hypo-osmotic regulation in these species. Recent work (Dehnel, 1962) has demonstrated that the osmoregulatory abilities of the two species changed significantly from summer to winter. The results presented here support and complement the latter findings with details of urine osmotic responses.

Effect of salinity

From an equilibrated or steady-state at time zero (Figs. 1 to 4), the urine osmotic response curves fall in low and rise in high salinities at rates which in general decline with time and reach new equilibria with media within the physiological limits of the species. Blood response curves for *Hemigrapsus* (Dehnel, 1962) and *Pachygrapsus* (Gross, 1957a) exhibit similar patterns. In the examples cited, most of the changes were complete by 24 hours' immersion in the media. In *Emerita*, an adjuster, Gross (1957a) showed that all blood changes were complete after only two hours in a comparable range of experimental salinities.

The antennary glands of *Pachygrapsus* have been shown to function mainly in the regulation of particular blood ions but not of total blood osmotic concentration (Jones, 1941; Robertson, 1949; Prosser, Green and Chow, 1955; Gross, 1957a, 1959). This conclusion was based on the isosmoticity of blood and urine in a variety of temperature and salinity combinations, and on high U/B ratios for magnesium (Gross, 1959). The prawns, *Palaemonetes varians*, *Leander serratus* and *L. squilla*, in dilute media produce urine isosmotic with blood (Panikkar, 1941). Parry (1954) showed Mg^{++} and SO_4^{--} to be lower in blood than in urine in *L. serratus.* Dehnel (unpublished data) has shown that the antennary glands of *Hemigrapsus* regulate the magnesium ion, which is similar to that reported for *Pachygrapsus.* In both species of *Hemigrapsus,* summer-adapted animals at least have total osmotic U/B ratios close to unity over the entire range of experimental temperature and salinity (Table I). At the same time, large osmotic gradients resulted in salinities below 75% sea water (Fig. 5) and the animals are regulating effectively. No significant weight increase was demonstrated in low salinities (Dehnel, 1962). After an initial rapid drop in urine concentration, the rate of salt loss diminished after 24 hours, and a new equilibrium resulted. Hyper-osmoticity of urine in summer animals exposed to concentrated media can have little adaptive importance, since salinities higher than 35% sea water are not as a rule encountered in this geographic area. Webb (1940) postulates that salt absorption is a continuous process under normal conditions. Hyper-osmotic urine in *Hemigrapsus* may be attributed to the suggested activity of salt-absorbing tissues in the gut and gills, which, when adapted to a high temperature and low salinity, continue to respond as in low salinities.

Three major differences distinguish the blood and urine osmotic responses of summer- and winter-adapted animals of both species, at their respective temperatures, to the range of experimental salinities from 12% to 150% sea water. The first is that over a series of sea water concentrations, winter animals showed a smaller range of urine concentration than summer animals. This was markedly true for *H. oregonensis* (Figs. 1 to 4). *Hemigrapsus nudus,* in winter, showed a reduced tolerance for very low external salinity. Such a reduction, expressed by high mortality, was also shown for *C. crangon,* a migratory shrimp (Broekema, 1941). The second difference was that U/B ratios for winter animals were in most cases significantly lower than summer ratios, because urine was considerably more hypo-osmotic to blood (Table I). This suggests winter participation of the antennary glands in hyper-osmotic regulation. The significance of low U/B ratios is not easy to see in relation to the third and most important difference between winter and summer responses: the production in winter of hypo-osmotic urine in external salinities above 75% sea water for *H. nudus* and above 100% sea water for *H. oregonensis.*

While hypo-osmotic regulation of blood concentration has been well documented for a number of Crustacea from aquatic, intertidal, semi-terrestrial and terrestrial habitats (Broekema, 1941; Jones, 1941; Prosser, Green and Chow, 1955; Gross, 1957a, 1957b; Riegel, 1959), it was not found in *Hemigrapsus* by Jones (1941), whose results have been cited widely. Gross (1957a), however, maintained that some degree of hypo-osmotic regulation of blood concentration occurred in *Hemigrapsus* from California, and gave a value of up to 33% perfect regulation for 20 hours in 150% sea water. Dehnel (1962) has shown that both species of *Hemigrapsus,* equilibrated in 75% sea water, did not maintain blood hypo-osmoticity when transferred to experimental salinities of 100% to 175% sea water. Present results have indicated that although hypo-osmotic regulation of blood was not established, increases in concentration may be resisted to some degree. It was shown that urine may differ in concentration from both blood and media, and that seasonal changes occurred in urine as well as blood osmotic responses.

Summer-adapted *Hemigrapsus* in the field were hyper-osmotic to summer salinities (25% to 35%) and blood and urine were nearly isosmotic. Similar osmotic behavior was found in *Carcinus* in dilute sea water and *Eriocheir* in fresh water (Krogh, 1939). Webb (1940) has suggested that active water uptake is suspended and ion exchanges in gills and antennary glands are intensified under these conditions. The low permeability characteristic of the exoskeleton of regulating forms would aid the animals in resisting the influx of excess water with increasing osmotic gradients (Gross, 1957a).

When exposed to increased or decreased experimental salinities, summer-adapted animals behaved osmotically as if they were still in "normal" summer conditions, although the concentration of their body fluids followed changes in the external medium. In low salinities, both species maintained hyper-osmoticity of blood and urine, and this might be accomplished as Webb (1940) suggested. Another possibility is that during the experimental period, salts are mobilized from adaptive extra-vascular pools, whose existence was postulated by Hukuda (1932) and verified in *Pachygrapsus* by Gross (1958, 1959). These pools in *Hemigrapsus* have not been established. Summer-type regulation, characterized by active ion absorption, and probably by reduced water intake, presumably accompanied by some selective ion reabsorption in the antennary glands, is, however, largely extra-renal and does not change after a period of 48 hours in experimental conditions.

The excretion of urine which is hypo-osmotic to the blood as a means of maintaining blood concentration above that of the medium is well documented. Winter-adapted *Hemigrapsus* in the field showed blood concentrations hyper-osmotic to 70% to 80% sea water, and *H. nudus* had higher blood concentrations than *H. oregonensis* in 75% sea water and 5° C. (Fig. 6). Urine data for winter animals from the field are not available, but after 51 hours (48 equilibration plus 3 experimental) in 75% sea water and 5° C. urine concentrations of the two species were similar and hypo-osmotic to the blood. For comparison with summer data, these values have been considered to approximate the urine and blood relationships in winter animals from field conditions.

Winter animals of both species, in experimental media below average winter sea water concentration, regulated their blood concentration with the production of hypo-osmotic urine. Blood and urine concentrations were significantly higher in *H. oregonensis* than in *H. nudus* in 12% and 25% sea water. Only *H. oregonensis* survived as long as 48 hours in 6% sea water (Figs. 2 and 4). The larger blood-to-medium gradients shown by *H. oregonensis* in 6%, 12% and 25% sea water suggest a more active ion-absorbing mechanism in this species, perhaps correlated with the animal's characteristically estuarine distribution. The active absorption of ions from hypo-osmotic media has been demonstrated in a variety of regulating Crustacea, among them, a crayfish, *Astacus*, and the crabs, *Carcinus* and *Eriocheir*, the latter being related to *Hemigrapsus* (Schwabe, 1933; Nagel, 1934; Krogh, 1939).

In crabs, the gills have been recognized as major sites of absorption (Nagel, 1934; Gross, 1957a; Green, Harsch, Barr and Prosser, 1959). Excess water can enter the animals through the gills by diffusion and by active absorption, together with specific ions. Urine, if formed by filtration, at first may be isosmotic with the blood and be rendered hypo-osmotic by the reabsorption of specific ions.

As long as ion loss in the urine is balanced by active absorption from dilute media, animals can achieve and maintain osmotic equilibrium. Increased urine output in dilute media has been shown to aid in elimination of excess water in *Carcinus* (Prosser and Brown, 1961). It has not been demonstrated in the present data but may be important in *Hemigrapsus* as well.

Effect of temperature

Broekema (1941) reported that *Crangon crangon* maintained in sea water of 29‰ showed a gradual decrease in blood concentration as experimental temperature was allowed to rise with the seasonal change from spring towards autumn (blood-medium gradient gradually increased). A reversal of these changes occurred when the experimental temperature was allowed to fall between autumn and winter. This species, in Dutch waters, winters offshore in water of relatively high salinity and migrates shoreward into more brackish conditions in spring and early summer. Survival at low temperatures was correlated with high salinity, and high temperature increased tolerance to low salinity. Other species, with a reverse migratory pattern, appeared to tolerate low salinities better at low temperatures. These included a spider crab, *Hyas araneus*, a shrimp, *Crangon allmani*, and a prawn, *Pandalus montagui*. A third group, represented by the crab, *Rhithropanopeus harrisi* and the amphipod, *Gammarus duebeni*, had tolerances similar to *Hyas* but did not migrate seasonally (Verwey, 1957). The two species of *Hemigrapsus* combine tolerances similar to *C. crangon*, and non-migratory habits. Dehnel (1960) suggested that low salinities at high temperatures may impose a greater stress than high ones. This is compatible with observed osmotic gradients maintained by these species between blood, urine and media in high and low salinities.

Urine and blood concentrations were alike for summer-adapted *H. oregonensis* in 12% sea water and 15° C. and the gradient between these fluids and the medium was 55% sea water. Cooling the animals at 5° C. reduced this gradient by 10% sea water for urine and 2% for blood. Blood osmotic concentration was regulated as strongly as at 15° C. Urine and blood concentrations were similar at 25° C., but the gradient between them and the medium increased, indicating that summer adaptation favors stronger regulation at high temperatures and low salinities and emphasizes the resemblance of the temperature and salinity tolerances of this species to those of *C. crangon*.

Summer-adapted *H. nudus*, at 5° C. in 25% and 12% sea water, showed urine to be significantly hypo-osmotic to blood (Table I), suggesting here also that the antennary glands are taking part in the elimination of excess water and reabsorption of ions. At 15° and 25° C., in dilute media, urine concentrations were not significantly different from those at 5° C., but blood-to-urine gradients were slightly reduced, suggesting that cooling of summer-adapted animals in low salinity conditions reduced their capacity for salt absorption and stimulated greater reabsorption in the antennary glands to compensate.

Winter-adapted animals of both species in dilute (12%) sea water showed significantly greater urine hyper-osmoticity at 5° than at 15° C. Blood data from winter-adapted animals at 12% and 5° C. are not available for comparison. With the rise in temperature, the U/B ratio decreased because absolute urine concentration decreased the greater. A rise in experimental temperature from 15° to 25° C. caused no further significant change in urine concentration. In 75% sea water, at 5° C., winter animals of both species showed similar urine concentrations but *H. nudus* had the higher blood concentration, hence a smaller U/B ratio resulted (Table I). At 15° C., *H. nudus* urine remained unchanged, blood concentration dropped, the U/B ratio rose, and regulation weakened. In *H. oregonensis*, however, blood did not change but urine concentration decreased, giving a lower U/B ratio. Thus, in experimental conditions approximating winter field temperature and salinity, *H. oregonensis* responded to a rise in temperature by a drop in urine concentration, while maintaining blood at the level found at 5° C. This is probably achieved by increased reabsorption in the antennary glands. A further rise in temperature to 25° C. caused no significant change in urine concentration. This species regulates less strongly in 75% sea water as temperature increases from 5° to 15° C. In *H. nudus*, the rise in temperature from 15° to 25° C. resulted in a significant decrease in urine, but not blood, concentration, hence a lower U/B ratio. U/B ratios for the two species in 75% sea water were identical at 25° C. but *H. nudus* had urine and blood values about 7% sea water higher than *H. oregonensis,* indicating somewhat stronger regulation.

The effects on urine concentration of cooling or warming summer-adapted animals and of warming winter-adapted animals were pronounced only in low experimental salinities. In high salinities, similar changes in temperature caused no significant change in urine concentration in either species. It is probable that high salinities pose less of an osmotic problem than low salinities, and that temperature changes consequently do not alter the balance between absorptive and reabsorptive activities as much in high as in low salinities.

SUMMARY

1. Total osmotic pressure measurements of urine were determined on two species of crabs, *Hemigrapsus nudus* and *H. oregonensis*, over a salinity range, 6% to 175% sea water, three temperatures, 5°, 15° and 25° C., and at two seasons, summer and winter. Blood data are included from Dehnel (1962) for comparison.

2. Urine and blood concentrations fall in dilute, and rise in concentrated media, at rates directly related to the gradients between media and equilibrated body fluid concentrations, and are influenced by the seasonal adaptation of the animals and the experimental temperature. Major changes in body fluids occurred within 48 hours.

3. Hyper-osmotic regulation in summer-adapted animals resulted in isosmoticity of blood and urine, implicating extra-renal mechanisms. The production of hypo-osmotic urine in winter-adapted animals indicated the participation of the antennary glands.

4. In both species, summer and winter adaptation tended to favor stronger hyper-osmotic regulation at the respective seasonal temperatures than at temperatures foreign to the seasons.

5. Changes in experimental temperature revealed seasonal and interspecific differences in 48-hour blood and urine concentrations. Blood concentrations of *H.*

oregonensis, when measured at a series of temperatures and salinities, showed a general trend, particularly for winter animals. As the concentration of the experimental media decreased (from 75% to 12%) blood concentrations increased significantly with decreasing temperature. Blood concentrations of summer animals showed no real differences, but when compared with winter crabs, at lower salinities, blood concentrations of summer crabs were significantly lower. The same general trend was shown for *H. nudus*. With respect to urine concentrations, summer-adapted *H. oregonensis*, in dilute media, showed significantly higher urine concentrations at higher temperatures. *H. nudus* showed no temperature effects in any salinities. Winter-adapted animals of both species showed significant decreases in urine concentration in low and intermediate, but not high, salinities, when the experimental temperature was increased.

6. Seasonal adaptation of osmoregulatory mechanisms in *Hemigrapsus* is shown to alter the balance of active processes so that for a given range of experimental conditions, urine is lower in winter animals than in summer, both in absolute concentration and relative to the blood.

LITERATURE CITED

Beadle, L. C., 1957. Comparative physiology: osmotic and ionic regulation in aquatic animals. *Ann. Rev. Physiol.*, **19**: 329–358.

Broekema, M. M. M., 1941. Seasonal movements and the osmotic behavior of the shrimp *Crangon crangon* (L.). *Arch. Néerl. Zool.*, **6**: 1–100.

Dehnel, P. A., 1960. Effect of temperature and salinity on the oxygen consumption of two intertidal crabs. *Biol. Bull.*, **118**: 215–249.

Dehnel, P. A., 1962. Aspects of osmoregulation in two species of intertidal crabs. *Biol. Bull.*, **122**: 208–227.

Green, J. W., M. Harsch, L. Barr and C. Ladd Prosser, 1959. The regulation of water and salt by the fiddler crabs, *Uca pugnax* and *Uca pugilator*. *Biol. Bull.*, **116**: 76–87.

Gross, W. J., 1954. Osmotic responses in the sipunculid, *Dendrostomum zostericolum*. *J. Exp. Biol.*, **31**: 402–423.

Gross, W. J., 1955. Aspects of osmotic regulation in crabs showing the terrestrial habit. *Amer. Nat.*, **89**: 205–222.

Gross, W. J., 1957a. An analysis of response to osmotic stress in selected decapod Crustacea. *Biol. Bull.*, **112**: 43–62.

Gross, W. J., 1957b. A behavioral mechanism for osmotic regulation in a semi-terrestrial crab. *Biol. Bull.*, **113**: 268–274.

Gross, W. J., 1958. Potassium and sodium regulation in an intertidal crab. *Biol. Bull.*, **114**: 334–347.

Gross, W. J., 1959. The effect of osmotic stress on the ionic exchange of a shore crab. *Biol. Bull.*, **116**: 248–257.

Gross, W. J., 1961. Osmotic tolerance and regulation in crabs from a hypersaline lagoon. *Biol. Bull.*, **121**: 290–301.

Gross, W. J., and P. V. Holland, 1960. Water and ionic regulation in a terrestrial hermit crab. *Physiol. Zoöl.*, **33**: 21–28.

Hukuda, K., 1932. Change of weight of marine animals in dilute media. *J. Exp. Biol.*, **9**: 61–68.

Jones, L. L., 1941. Osmotic regulation in several crabs of the Pacific Coast of North America. *J. Cell. Comp. Physiol.*, **18**: 79–92.

Krogh, A., 1939. Osmotic Regulation in Aquatic Animals. Cambridge at the University Press.

Lockwood, A. P. M., 1962. The osmoregulation of Crustacea. *Biol. Rev.*, **37**: 257–305.

Nagel, H., 1934. Osmoregulation in Crustacea. *Zeitschr. vergl. Physiol.*, **21**: 468–491.

Panikkar, N. K., 1941. Osmotic behavior of fairy shrimp, *Chirocephalus*. *J. Exp. Biol.*, **18**: 110–114.

Parry, G., 1954. Ionic regulation in the palaemonid prawn *Palaemon* (=*Leander*) *serratus*. *J. Exp. Biol.*, **31**: 601–613.

Peters, H., 1935. Über den Einfluss des Salzgehaltes im Aussenmedium auf den Bau und die Funktion der Exkretionsorgane dekapoder Crustaceen. (Nach Untersuchungen an *Potamobius fluviatilus* und *Homarus vulgaris*.) *Zeitschr. Morph. Okol. Tiere*, **30**: 355–381.

Picken, L. E. R., 1936. The mechanism of urine formation in invertebrates. I. The excretion mechanism in certain arthropods. *J. Exp. Biol.*, **13**: 309–328.

Prosser, C. L., and F. A. Brown, Jr., 1961. Comparative Animal Physiology. Philadelphia, W. B. Saunders Co.

Prosser, C. L., J. W. Green and T. J. Chow, 1955. Ionic and osmotic concentration in blood and urine of *Pachygrapsus crassipes* acclimated to different salinities. *Biol. Bull.*, **109**: 99–107.

Riegel, J. A., 1959. Some aspects of osmoregulation in two species of sphaeromid isopod Crustacea. *Biol. Bull.*, **116**: 272–284.

Robertson, J. D., 1949. Ionic regulation in some marine invertebrates. *J. Exp. Biol.*, **26**: 182–200.

Robertson, J. D., 1953. Further studies on ionic regulation in marine invertebrates. *J. Exp. Biol.*, **30**: 277–296.

Schwabe, E., 1933. Über die Osmoregulation verschiedener Krebse (Malacostracen). *Zeitschr. vergl. Physiol.*, **19**: 183–236.

Verwey, J., 1957. A plea for the study of temperature influence on osmotic regulation. *Ann. Biol.*, **33**: 129–149.

Webb, D. A., 1940. Ionic regulation in *Carcinus maenas*. *Proc. Roy. Soc. London, Ser. B*, **129**: 107–136.

Wikgren, Bo-Jungar, 1953. Osmotic regulation in some aquatic animals, with special reference to the influence of temperature. *Acta Zool. Fennica*, **71**: 1–102.

THE ADAPTATION OF TETRAHYMENA TO A HIGH NaCL ENVIRONMENT[1]

PHILIP B. DUNHAM

Department of Zoology, University of Chicago, Chicago, Ill.[2]

Many groups of animals have successfully adapted to an osmotically altered environment or osmotic stress. Response and adaptation to a different osmotic and ionic environment have enabled representatives of all major phyla except the echinoderms to invade fresh water from the sea. Other examples are invasion of the sea by fresh-water teleosts, adoption of fresh water for larval development by terrestrial insects, and return to fresh water by terrestrial pulmonate snails. Similar osmotic and ionic problems are faced by estuarine animals and by animals migrating from fresh water to the sea (or *vice versa*) to breed. The problem in these latter instances is met anew by single individuals, as well as over many generations. (For further discussion, see Krogh, 1939.)

The present study was concerned with the adaptation to an osmotic stress by *Tetrahymena pyriformis*, a ciliate which normally lives in fresh water. The stress was introduction into a high NaCl environment. The process of adaptation was investigated from the initiation of the stress through many generations. There appeared to be selection for stress tolerance at the first generation after the stress, and selection for ability to regulate NaCl over many generations. Inorganic ion regulation in the normal and adapted cells was investigated and compared.

Tetrahymena is suited to a study of this nature for several reasons:

(1) It has been known for some time that *Tetrahymena* can adapt to an environment much more concentrated than fresh water (Chatton and Tellier, 1927; Loefer, 1939).

(2) Large homogeneous quantities of cells in suspension needed for physiological studies could be grown easily and quickly.

(3) Studies encompassing many generations are feasible since *Tetrahymena* has a short generation time.

(4) Osmotic regulation by cells is probably effected in large part by controlling particular inorganic ions (*cf.* Brown and Stein, 1960). The main features of inorganic ion regulation in normal (unadapted) *Tetrahymena* are known (Dunham and Child, 1961). *Tetrahymena*, like other fresh-water protozoa and cells of lower fresh-water invertebrates, maintains itself hyper-osmotic to its environment, and also maintains remarkably constant potassium and sodium concentrations over a wide range of hypo-osmotic environmental concentrations. *Tetrahymena* maintains in normal environments a higher potassium concentration than sodium or

---

[1] The author gratefully acknowledges the guidance of Dr. H. Burr Steinbach throughout this study. Supported by National Science Foundation grant G-4526 and United States Public Health Service training grant 2G-150.

[2] Present address: Department of Zoology, Syracuse University, Syracuse, N. Y.

chloride, sodium being more concentrated than in the environment in very dilute environments. *Tetrahymena* extrudes sodium into more concentrated environments, but the extrusion is maximal at a relatively low external sodium concentration (20 m*M*). Potassium is accumulated actively and independently of sodium extrusion. *Tetrahymena* has limited ability to regulate its ion concentration in hyperosmotic environments. These findings have been corroborated by Andrus and Giese (1963).

There is no general agreement on the use of terms pertaining to adaptation and related phenomena. For the purposes of this study, the following terms and definitions are used. The first response of an individual organism to an environmental stress is called the *immediate response* (*cf.* Prosser, 1958). The individual is not changed in any way that is not readily reversed by removing the stress.

The second response, occurring after a longer exposure of an animal to stress, is called *acclimation* (*cf.* Prosser, 1958). This involves compensation in which case the individual is changed in ways that would not readily revert to the original condition upon removal of the stress. As Prosser (1958, p. 168) put it, there is "a new equilibrium of rate functions." Immediate response and acclimation do not involve changes in the genetic constitutions of the individual and therefore are not inherited (though they are certainly a reflection of the genetic makeup of the individual).

A population which has survived an environmental stress for at least one generation may be different from the population of the previous generation, the changes being associated with changes in the gene pool of the population. The stress in this case is *selection pressure*, which may be defined as all causes of systematic and heritable change in populations between one generation and the next that do not directly involve mutation or introduction (or loss) of genetic material from outside (or out of) the population (*cf.* Simpson, 1953, p. 138, and Lerner, 1958, p. 5).

Adaptation is the acquisition within a population of heritable advantageous (adaptive) characteristics, resulting from selection, mutation, introduction into the population, etc. Adaptation may suit a population to an altered environment, or improve the relation of a population to a constant environment.

Adaptation resulting from selection of initially more tolerant individuals is called *preadaptation*. Preadaptation has been defined as the "chance adaptive effects of variation" in a population (Emerson, 1949, p. 642).

Heritable variability exists in every natural population, including those comprised of asexually produced descendants from one ancestor, according to Simpson (1953, pp. 61 and 65).

Adaptation or acclimation to osmotic and ionic stress under quantitatively defined conditions has been reported for a number of animals. Important examples are *Cordylophora* (Kinne, 1958), mosquito larvae (Wigglesworth, 1938), grapsoid crabs (Gross, 1961), the crab-eating frog (Gordon *et al.*, 1961), the eel (Keys, 1933; Krogh, 1939), a number of ciliates (Gause, 1941 and 1942; Loefer, 1939; see Loefer for earlier references), and *Amoeba mira* (Mast and Hopkins, 1941). For additional examples and references, see Prosser (1955) and Bullock (1960).

Preliminary reports of the present work have been published (Dunham, 1961 and 1962).

METHODS AND MATERIALS

Tetrahymena pyriformis strain W, an asexual amicronucleate strain, was cultured axenically in 2% proteose-peptone (normal medium) or in 2% proteose-peptone + 1% NaCl (high NaCl medium). One-liter Roux bottles containing 500 ml. of medium were inoculated with 5 ml. of medium of a culture in the log phase of growth. Cells were harvested by gentle centrifugation after 4–6 days' growth (normal animals) or 9–12 days' growth (adapted animals) at 22–24° C.

Adapted cultures were started by transferring normal animals with a platinum loop directly into the high NaCl medium without culturing through media of intermediate NaCl concentrations. Failure of adapted cultures to appear was rare.

Some aspects of the process of adaptation were investigated by observing single cells introduced into drops of media of several NaCl concentrations at 23° C. Parameters observed were immediate osmotic response, subsequent recovery, and cell division. NaCl concentrations in the media were 35 m*M*, 120 m*M*, or 200 m*M*. The media also contained 5 m*M* KCl, 0.5 m*M* $MgCl_2$ (K and Mg are growth requirements of *Tetrahymena;* Kidder *et al.*, 1951), and 0.1% proteose-peptone. Drops about 5 mm. in diameter could be kept under mineral oil indefinitely without evaporation. Clones developed and remained "healthy" for as long as a month under these conditions. No special measures were taken to keep the clones axenic.

Methods for packed cell volumes, dry weights of packed cells, number of cells per unit volume, preparation of cell extracts, and analyses of K, Na, and Cl concentrations in cell extracts and media, were as previously reported (Dunham and Child, 1961). Total exchangeability and kinetics of exchange of intracellular Na were determined using Na^{24}. The isotope was obtained from Oak Ridge National Laboratories as NaCl in HCl solution, and was neutralized with NaOH before use. Trace amounts of Na^{24} were added to cell suspensions, samples were removed periodically, and the cells were spun down. The experiments were never complicated by concomitant net fluxes of Na. Counts per minute of wet samples of medium and cell extracts were determined with a Geiger-Muller detector of a NaI-Tl crystal scintillation well detector. All counts were greater than six times background. Per cent exchanges of intracellular Na were calculated from the specific activities of the medium and of the cells, after appropriate corrections for extracellular space.

Extracellular spaces of packed cells were determined using C^{14}-inulin, added to cell suspensions within 30 seconds prior to centrifugation. Radioactivity of dried samples of the supernatant and of cell extracts was determined in a gas flow, windowless counter. Total inulin concentration was always less than 0.05%, which is negligible osmotically.

RESULTS

Process of adaptation

The method was described above for studying the process of adaptation by observing immediate response and clone formation of cells in drops of medium under mineral oil. The results of these experiments are collected in Table I. The controls for this series of experiments were performed as follows: (A) Single normal cells were placed in drops of 35 m*M* NaCl medium, and (B) single adapted cells were placed in drops of 200 mM NaCl medium. All cells survived to divide, showing that failure to do so cannot be attributed to handling of the cells, or to deficiencies in the media. In all experiments described in this section, a successful clone (taken as at least 32 animals, the product of 5 or more generations) never failed to develop if a cell survived to divide once. Therefore the number of surviving cells was always the same as the number of successful clones formed.

(C) Normal cells were introduced singly into drops of 200 mM medium. Within seconds all cells became flattened, due to osmotic loss of water. After 30 minutes, all cells had swelled somewhat. Most cells were nearly immotile. After three hours, only the few cells which eventually survived to divide were still motile. Division occurred from 10 to 30 hours after introduction into the 200 mM medium. (Normal generation time was 3–4 hours; see below.) No mortality was observed in the subsequent several generations. After the first division, the cells were smaller and more nearly spherical than the original cells.

Table I

Survival of single normal and adapted Tetrahymena introduced into drops of 35 mM or 200 mM NaCl medium under mineral oil. Results are expressed as per cent survival, i.e., per cent of cells which survived to divide. Clones developed from all cells which survived to divide once

Experiment	Number cells introduced	Number cells surviving	Per cent survival
(A) Normal cells into 35 mM NaCl medium.	36	36	100
(B) Adapted cells into 200 mM NaCl medium.	70	70	100
(C) Normal cells into 200 mM NaCl medium.	702	16	2.3
(D) Normal cells equilibrated in 120 mM NaCl medium, then into 200 mM NaCl medium.	36	26	72
(E) Cells from (C) and (D), after 8 generations, into 35 mM medium. C)	99	98	99
D)	35	35	100
(F) Cells from (C) and (D), after 8 generations, into 35 mM NaCl medium for 2 hours, then returned to 200 mM NaCl medium. C)	35	33	94
D)	36	13	36

(D) Normal cells were equilibrated in 120 mM medium for 30–45 minutes. No mortality was observed. (No attempt was made to obtain quantitative data on this point.) These cells were then transferred singly to drops of 200 mM medium.

(E) Cells from clones from experiments (C) and (D), after 8 generations in 200 mM NaCl medium, were introduced into 35 mM medium. No significant mortality was observed. This experiment served as a control for experiment (F).

(F) Cells from clones from experiments (C) and (D), after 8 generations in the 200 mM medium, were introduced in 35 mM medium, allowed to equilibrate for two hours, then returned to the 200 mM medium.

Cells from (C) are descendants of cells which survived the stress of direct transfer before acclimating to the high NaCl medium. Cells from (D) are descendants of cells which acclimated to high NaCl medium, but most likely would not have survived the stress of direct transfer, since experiment (C) showed that only 2% of the population were tolerant of the stress. Experiment (F) shows that many more descendants of survivors from (C) were tolerant of stress than were descendants of cells from (D). These results suggest a heritable difference between normal and adapted cells.

General morphological and physiological characteristics

Average cell volume for the adapted culture was 9.5 $\mu\mu$l. ± 0.47 (S.E., 5 determinations; determined from packed cell volumes, cell counts, and extracellular spaces). Cell volume did not change during the 22 months the adapted culture was investigated. The average cell volume of normal animals was previously reported as 1.83×10^{-5} μl., or 18.3 $\mu\mu$l. (Dunham and Child, 1961). A more recent determination gave 16.0 $\mu\mu$l., and this value will be used in the present report. (Average cell volumes for *T. pyriformis* strain GL reported by various authors, cited by Zeuthen, 1963, ranged from 16 to 25 $\mu\mu$l.) Adapted cells were not only 40% smaller than normal cells, they also had a different shape, being more nearly spherical than the normal cells. This difference is indicated by width:length ratios of cells of the two types measured on phase micrographs of cells from early stationary phase cultures. The width:length ratios were about 0.5 for normal cells and 0.75 for adapted cells.

The per cent dry weight of adapted cells was 28.8% ± 0.53 (S.E., 5 determinations). The per cent dry weight of normal cells was previously found to be 19.4% (Dunham and Child, 1961). This value has recently been confirmed (19.3%).

A reliable difference between the densities of cells of the two cultures was not distinguished with the methods used. It might be expected on the basis of the difference in per cent dry weights that the adapted cells would have a density 3–4% higher than that of the normal cells. However, assuming the densities of the two cell types were both in the range generally observed (1.05–1.10 g./ml.), the average dry weight per cell was calculated to be 3.2–3.4 mμg. per normal cell and 2.9–3.0 mμg. per adapted cell. Therefore, even though the per cent dry weight of adapted cells is 33% greater than that of normal cells, the amount of non-volatile material per cell is very nearly the same. These values for dry weight per cell are similar to those reported elsewhere. Scherbaum (1957) found 4.1 mμg. for *T. pyriformis* strain GL in "normal mass culture." Hamburger and Zeuthen (1960) found 2–4 mμg. dry weight per cell for strain GL, depending upon the phase of growth of the culture.

In order to determine generation times, numbers of divisions in 36 hours were counted in at least 30 clones in drops under oil. Generation time for adapted cells was about 11 hours at 23° C. Cells adapted for only a few generations and for 22 months (about 1500 generations) had nearly the same generation times. For normal cells the generation time was 3–4 hours at 23° C. (Scherbaum and Zeuthen, 1955, reported 2.3 hours generation time for *T. pyriformis* strain GL in 2% proteose-peptone at 28.5° C., the optimum temperature for growth.)

The adapted animals had a much lower motility than the normal animals. However, if a suspension of adapted animals in high NaCl medium was diluted with a sucrose solution isosmotic with the high NaCl medium, the motility of the cells increased immediately, indicating that the high NaCl medium imposes an ionic as well as an osmotic stress.

TABLE II

Ion concentrations, in mM/l., of normal medium and high NaCl medium

	Na	K	Cl
Normal medium	36.5	5	28.7
High NaCl medium	223	5	215

Extracellular space

Per cent extracellular space of packed adapted cells, in media with Na concentrations less than 300 m*M*, was 13.3% ± 0.5 (S.E., 34 determinations). The full range was from 9% to 17%. Extracellular space seemed to be minimal (9–11%) in media with Na concentrations of 130–170 m*M*, and slightly higher on either side of this range. In media with Na concentrations greater than 300 m*M*, extracellular was around 20%. The shapes of the cells are different in various osmotic conditions, being swollen in dilute media, and shrunken and wrinkled in more concentrated media. The variations in extracellular space are no doubt due to differences in packing of the variously shaped cells.

The mean per cent extracellular space of packed normal cells in normal medium was 9.8%, and in high NaCl medium, 15%. Use of radioactive iodinated serum albumin (Risa) for determining extracellular space gave higher values than did using C^{14}-inulin. For normal cells in normal medium, it was 15% (Dunham and Child, 1961). Delaying this centrifugation after adding Risa resulted in increased extracellular space. This shows the cells take up the Risa.

When using C^{14}-inulin, values for per cent extracellular space were not increased by delaying centrifugation. Therefore, no error resulted from uptake of inulin. (Kidder and Dewey, 1945, showed that *T. pyriformis* does not utilize inulin.) Adding unlabeled inulin to a concentration 10-fold greater than that of the C^{14}-inulin had no effect on per cent extracellular space. Therefore, no error results from binding of inulin by cell surfaces.

Unless otherwise indicated, all cellular ion concentrations in this paper have been corrected for extracellular space.

Ion regulation

The cellular sodium concentration (Na_i) in normal *Tetrahymena* in normal medium was 12.7 meq./l. of cells. In normal *Tetrahymena* equilibrated (30 min-

TABLE III

Cellular sodium concentrations (Na_i) in adapted Tetrahymena in high NaCl medium. Time periods show months during which analyses were made after introduction into high NaCl medium. Mean, standard error of the mean, and number of determinations are given

Stage	Time period (months)	Na_i (meq./l. cells)
I	1–2	42.8 ± 2.02 (5)
II	6–7	35.5 ± 2.92 (9)
III	10–12	27.0 ± 1.22 (5)
IV	18–22	21.1 ± 0.75 (15)

utes) in high NaCl medium, Na_i was about 105 meq./l. cells (Dunham and Child, 1961). Ion concentrations of these media are given in Table II. Cells of the adapted culture were first analyzed for Na_i two weeks after introduction into the high NaCl medium, and periodically over the subsequent 22 months. Mean values, grouped in four time periods, are shown in Table III. These data show that after two weeks of adaptation, Na_i had been lowered to 60% less than Na_i in normal cells equilibrated a short time in high NaCl medium. Na_i continued to decrease to 80% less than the initial high level.

Adapted cells were equilibrated 30–60 minutes in various dilutions of high NaCl medium, and in media with increased NaCl concentrations. These experiments were performed on cells between stages I and II and in stage IV of adapta-

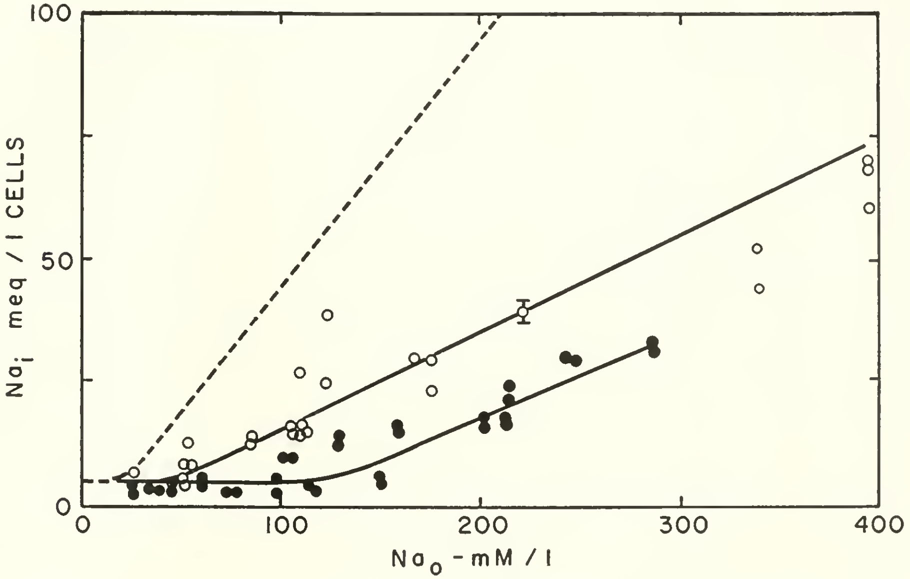


FIGURE 1. Na_i (in meq./l. cells) in normal *Tetrahymena* (dotted curve) and in adapted *Tetrahymena* in stage I–II of adaptation (open circles) and in stage IV (solid circles). Brackets around point for stage I–II cells at Na_o of 227 mM indicate standard error of a mean for 12 determinations. All other points represent single determinations after equilibration in media of various Na_o. Curves were fitted by eye.

tion (see Table III). Na_i in adapted cells in high NaCl medium between stages I and II was 39.4 meq./l. cells ± 1.9 (S.E., 12 determinations). Na_i from these experiments is plotted against Na_o (external sodium concentrations) in Figure 1. Included in this figure are comparable data (dotted curve) for normal *Tetrahymena* (from Dunham and Child, 1961).

Na_i in all three kinds of cells in Figure 1 was constant at 3–5 meq./l. in the lower ranges of Na_o. In normal cells Na_i was constant in external Na concentrations up to about 20 mM; in stage I–II adapted cells up to 45 mM; and up to about 125 mM in stage IV cells.

Above these levels of Na_o, Na_i increased roughly linearly with Na_o in all three cell types. The approximate slopes of these linearly increasing portions of the curves were: normal, 0.5; stage I–II, 0.2; stage IV, 0.2.

K_i in adapted cells did not change significantly during the 22 months the culture was studied. Mean K_i was 33.3 meq./l. cells ± 0.7 (S.E., 62 determinations). K_i in normal cells was 31.7 meq./l. cells (Dunham and Child, 1961). Calculated in terms of amount of K per unit number of cells, K_i in adapted cells was 31.6 μeq./10^8 cells and 50.7 μeq./10^8 cells in the normal culture. Whereas the amounts of K per cell are very different, the concentrations per cell volume are very nearly the same in the two cultures.

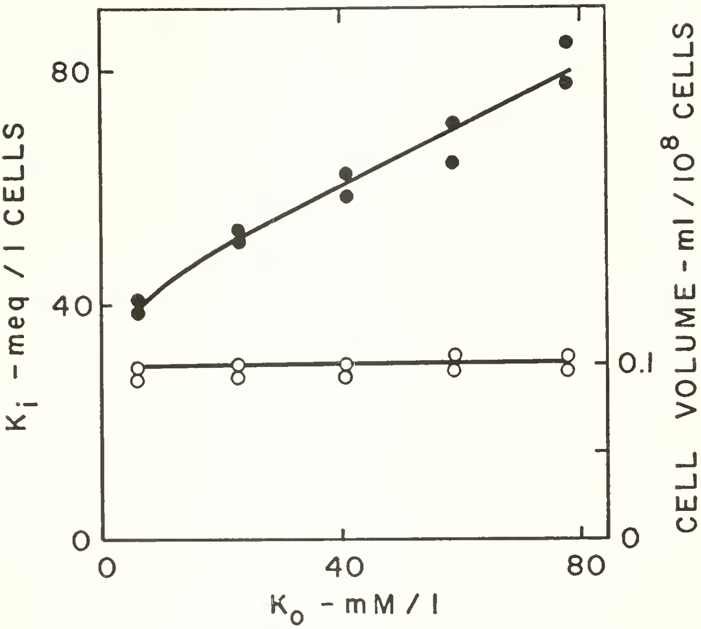


FIGURE 2. K_i in adapted *Tetrahymena* equilibrated 90 minutes in high NaCl media with various increased K_i concentrations. K (solid circles) in meq./l. cells; cell volume (open circles) in ml./10^8 cells.

K_i in adapted cells readily increased with increased K_o. Figure 2 shows K_i in adapted cells equilibrated for 90 minutes in media to which various amounts of KCl had been added. The slope of the linearly increasing portion of the curve is 0.49. The slope of the comparable curve for normal animals was 0.52 (Dunham and Child, 1961).

Figure 3 shows values for Cl_i for adapted cells equilibrated in dilutions of high NaCl medium, and in media made more concentrated with respect to Cl by adding NaCl or KCl. These values are not corrected for extracellular space. No adapted cells were analyzed for Cl_i prior to stage III of adaptation (see Table III). There was no significant difference between stage III and stage IV cells. Below Cl_o of about 125–140 m*M*, the points for Cl_i fall about a line with a slope of 0.1. The extracellular space fractions in this range of medium concentrations were between 0.09 and 0.12. Therefore, the slope of this line is due to extracellular Cl, and intracellular Cl is constant below 125–140 m*M* Cl_o at about 2.5 meq./l. cells.

Above Cl_o of 125–140 m*M*, uncorrected Cl_i increases with Cl_o with a slope greater than can be accounted for by extracellular Cl. There is considerable scatter of the points, but the slope is approximately 0.2, or, corrected for extracellular space, 0.07. The dotted curve in Figure 3 shows Cl_i corrected for extracellular space according to the above considerations. So Cl regulation in adapted cells corresponds qualitatively to Na regulation, but the constant Cl_i level below Cl_o of 125 m*M* is less than the constant Na_i level below Na_o of 125 m*M*, and slope of the increasing portion of the Cl curve is considerably less than in the case of Na. Figure 3 shows that the relationship between Cl_i and Cl_o is the same whether Cl_o is increased by adding NaCl or KCl to the medium.

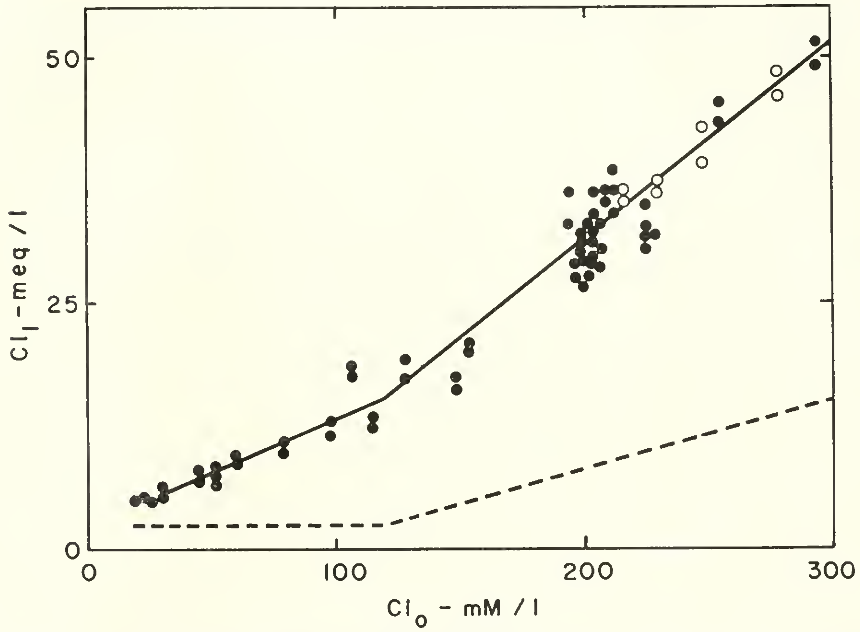


FIGURE 3. Cl_i in adapted *Tetrahymena* in high NaCl medium, equilibrated in dilutions of high NaCl medium, and equilibrated in media with Cl_o increased by adding NaCl (solid circles) or KCl (open circles). Values uncorrected for extracellular space. Curves were fitted by eye. Dotted curve shows data corrected for extracellular space (see text).

Figure 4 shows Cl_i in normal cells equilibrated in dilutions of normal medium, and in media made more concentrated with respect to Cl_o by adding NaCl or KCl (Cl_i values uncorrected for extracellular space). The mean of 14 determinations of Cl_i in cells in normal medium was 6.6 meq./l., or corrected for extracellular space, 4.1 meq./l. cells. The constant level in dilute medium is greater in normal cells than in adapted cells. The region of Cl_o in which Cl_i begins increasing with Cl_o is much lower (40–50 mM) in normal cells than in adapted cells, and the slope of the increasing portion of the curve is much greater in normal cells (corrected slope, approximately 0.3).

Kinetics of net changes of intracellular Na and K in normal cells upon changes in external ion concentrations have been reported (Dunham and Child, 1961). Na_i and K_i readily increased upon increase of Na_o and K_o, respectively. Na_i readily decreased, but K_i only very slowly, upon dilution of the medium. Subsequent experiments have shown that Cl_i readily increases or decreases upon appropriate changes of external concentration (unpublished).

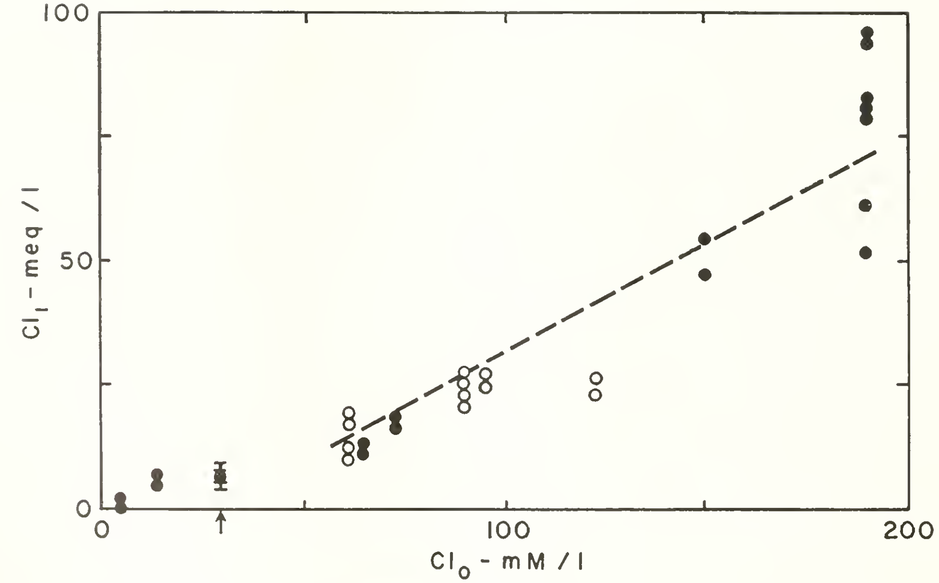


FIGURE 4. Cl_i in normal *Tetrahymena* equilibrated in dilutions of normal medium, in normal medium (arrow on abscissa), and equilibrated in media with Cl_o increased by adding NaCl (solid circles) or KCl (open circles). Values uncorrected for extracellular space. Brackets around point in normal medium show standard error (inner brackets) and standard deviation (outer brackets) for 14 determinations. Curve eye-fitted.

Table IV shows further experiments on rates of changes of cellular ions upon changes of ions in the medium. Experiments A and B in Table IV indicate that adapted cells are freely permeable to Na and Cl.

In experiment A, K_i remained virtually constant. Na_i increased rapidly in the first minute, and then slowly over the next two hours. The cells shrank in the first minute to at most 65% of initial volume, swelled in the next half hour to 78% of initial volume, and remained constant.

In experiment B in Table IV, K_i had decreased somewhat by 50 minutes to 85% of the initial amount of K per cell. Net Na efflux was complete in 15 minutes, whereas Cl_i fell to nearly zero within two minutes.

TABLE IV

Kinetics of net changes of intracellular ions in Tetrahymena upon dilution of or addition of NaCl to the medium

Experiment	Time, minutes	Cell ion content, meq./10^8 cells			Cell volume, ml./10^8 cells
		K	Na	Cl	
(A) Adapted cells. Na_0, initially 225 m*M*, increased to 396 m*M* at zero time.	0	41.7	35.2	—	0.95
	1	38.6	43.2	—	0.62
	27	41.1	44.7	—	0.74
	130	37.1	51.1	—	0.74
(B) Adapted cells. High NaCl medium diluted four-fold at zero time.	0	38.6	36.4	17.3	1.14
	2	38.8	20.2	0	2.00
	15	29.1	6.3	0	1.57
	50	32.5	5.2	0	1.43
(C) Normal cells. Equilibrated 45 minutes in normal medium with NaCl concentration increased by 170 m*M*. At zero time this medium diluted with normal medium to a NaCl concentration of about 62 m*M*.	0	38.9	100	102	0.89
	1.5	41.1	52.2	35.5	1.47
	20	31.6	27.5	9.8	1.23

The kinetics of net influx of Na after Na_0 is increased were of the same general order in normal and adapted cells. Net efflux of Na upon dilution of the medium was slower from adapted cells than from normal cells. However, when normal cells were first equilibrated in high NaCl medium, then net efflux of Na upon dilution of the medium is slower, comparable to the rate of efflux from adapted cells. Experiment (C) in Figure 4 demonstrates this point. These results indicate that permeability to Na is comparable in normal and adapted *Tetrahymena.* These data on fluxes were not sufficient for critical calculations of flux rates.

The exchangeability of Na_i with Na_0 in adapted cells was determined using Na^{24} as a tracer. The data are given in Table V. About 70% of Na_i was available for exchange. The amount of non-exchangeable Na (10–11 meq./l. cells) as determined with the tracer is greater than the constant Na_i component of 3–5 meq./l. cells shown in Figure 1. Apparently some intracellular Na which does

TABLE V

Exchangeability of Na_i with Na^{24} in adapted Tetrahymena. Mean per cent exchange (relative specific activity), standard error of the mean, and number of determinations are given. Experiments carried out on stage II cells in high NaCl medium

Time (minutes)	Per cent exchange
1	18.3 — (2)
13–16	51.3 ± 7.4 (5)
95–115	73.6 ± 3.8 (5)
225–250	69.7 ± 17.9 (5)
350	67.9 — (1)

not readily exchange with Na^{24} is nevertheless mobilized when the cells are placed in very dilute medium. Otherwise, the Na not removed from the cells in dilute medium is also probably non-exchangeable.

DISCUSSION

The results of this study might best be considered within the framework of a "microevolutionary" process (*cf.* Lerner, 1958, p. 4). The results can be organized in terms of several conditions and events of this process.

(1) The immediate response to the stress of transfer to high NaCl was observed both in quantitative experiments and by observing cells under the microscope. There was a volume decrease of at least 50% during the first minute, due to osmotic loss of water. There was then a net influx of Na, and a concomitant reentry of water and volume increase. These are passive changes, and involve no compensation by the animal.

(2) The data indicate preadaptive variability in the original population. Two per cent of the cells were sufficiently tolerant of the stress to survive and divide. Seventy-two per cent of the normal population can survive NaCl if they are first equilibrated in an intermediate NaCl concentration. Therefore, the characteristic which distinguished the 2% from the rest of the population is tolerance of stress, and not ability to acclimate to high NaCl.

The data also suggest that stress tolerance was a heritable characteristic. Almost all (94%) descendants of the cells which originally survived stress of direct transfer could also survive a comparable stress. On the other hand, consider cells living in high NaCl after the stress was minimized by equilibration in an intermediate NaCl concentration. A much lower proportion of the descendants of these cells could survive the stress of transfer to high NaCl. Therefore, it may be concluded that cells tolerant of stress pass this character to their descendants.

(3) The high NaCl medium to which the normal cells are transferred represents a greatly increased selection pressure due to osmotic and ionic stress. The survival and reproduction of a small segment of the population represents selection since the resultant population differed genetically from the original one in that nearly 100%, rather than 2%, of its members carried the character for stress tolerance.

(4) The gradual decrease in cellular Na concentration in the adapted cells constituted further adaptation over a long time. In this way, the population became better suited to a constant environment. Most likely there was variability in ability to regulate salt, and the better regulators had some selective advantage, either lower mortality rate or shorter generation time. These better suited animals became relatively more prevalent through selection, and average cellular Na concentration decreased. Selection for heritable characters is a reasonable explanation for this phenomenon, particularly when it is considered that it took place over the course of about 1500 generations (22 months, 11 hours per generation).

The question might be raised, whether these characters, stress tolerance and salt regulatory ability, are passed from one generation to the next by nuclear or by cytoplasmic inheritance. In an asexual organism, no simple test, short of nuclear transplantation, could give an unequivocal answer to this question. However, inheritance of some cytoplasmic characters may be just as stable and as significant as nuclear inheritance. According to Simpson (1953, p. 83), "Cytoplasmic inheritance has little effect sharply separable from nuclear inheritance."

(5) There was acclimation (compensatory changes at the level of the individual rather than the population) very soon after introduction into high NaCl. Some changes must have taken place before the first division. First of all, the first division was delayed up to 30 hours. Second, the first division was critical for the formation of a new population. No mortality was observed in the subsequent generations, but there was considerable mortality before the first division. And third, the cells were smaller and more nearly spherical after the first division.

Since the amount of dry material per cell was nearly the same for the normal and adapted cells, the smaller volume of the adapted cells is due to a difference in amount of water per cell. So the adapted cells are not simply a smaller, stunted version of the normal cells, but are of altered composition. From one cell division to the next there is the same amount of organic synthesis in the two cell types. The longer generation time of adapted cells might reflect a diversion of energy from growth processes to ion regulation. Toxic effects of the higher salt concentration on metabolism are another possibility.

The simple morphological differences between the two strains (difference in size, shape, and composition) probably are functionally related to the adaptation. Similar changes in other animals have been taken as sufficient criteria for acclimation or adaptation (in *Cordylophora*, by Kinne, 1958; in mosquito larvae, by Wigglesworth, 1938). In the present study, morphological and physiological changes were observed concomitantly. More work is needed to establish the exact relationship between the various types of change. Preliminary studies comparing the electrophoretic patterns of soluble proteins from normal and adapted cells were done in conjunction with the present study. The patterns were identical with the exception of one protein which was in adapted cells in amounts an order of magnitude greater than in normal cells. (For techniques employed, see Crockett, Dunham and Rasmussen, 1964.)

The primary physiological change arising in the course of adaptation was increased capacity to maintain low cellular concentrations of Na and Cl.

Both normal and adapted *Tetrahymena* were permeable to Na and Cl since net fluxes of these ions in or out of cells were observed after changing the external concentrations of Na and Cl. Also, most intracellular Na readily exchanged with Na^{24}.

Active Na transport has been demonstrated in a variety of tissues in a number of animals, both as extrusion from cells, in such systems as human erythrocytes, frog muscle, and squid nerve, and as transcellular transport, in rabbit kidney, toad urinary bladder, and frog skin (see review by Andersen and Ussing, 1960, for references). Na extrusion is probably characteristic of all animal cells.

Na extrusion by *Tetrahymena* was indicated by the constant maintenance of Na over a range of Na_o up to a level of Na_o considerably greater than Na_i. This region of Na_o in which Na_i begins increasing with increased Na_o represents the lowest level of Na_o at which Na extrusion operates maximally, *i.e.*, the saturation level of the Na extrusion mechanism (Dunham and Child, 1961). Na extrusion in *Tetrahymena* has also been demonstrated by Andrus and Giese (1963). In adapted

Tetrahymena this saturation level gradually increased over 1500 generations, to about 6 times the saturation level in normal cells.

A constant level of Na_i is maintained in normal and adapted cells in media with Na_o below the saturation level of Na extrusion. Presumably this constant Na level is a cellular Na component which remains constant even as total Na_i increases in media with Na_o above the saturation level. The mobile Na_i component (Na_i in addition to the constant component) readily underwent net changes, and was linearly related to Na_o. Therefore, mobile Na_i is probably in equilibrium with Na_o (taking into account the gradient imposed by Na extrusion, which is constant above the saturation level) with a constant mobile $Na_i:Na_o$ ratio. The slopes of the linearly increasing portions of the Na_i/Na_o curves must then be functions of the steady-state between Na_o and mobile Na_i.

This slope is called here "apparent free Na space." In normal cells, the "apparent free spaces" for Na and K were the same, suggesting that the slopes were a measure of the fraction of cell volume available for equilibration with the medium, *i.e.*, the osmotically active fraction of cell volume (Dunham and Child, 1961). However, additional data necessitated rejection of this hypothesis. First, "apparent free Na space" decreased with adaptation, but "apparent free K space" was unchanged. Second, "apparent free Cl space" is less than either Na or K "space" in normal and adapted cells.

Therefore "apparent free spaces" represent physiological characteristics of the cells which are specific for each ion species. In particular, the changes in "apparent free spaces" for Na and Cl with adaptation are compensations directed specifically toward regulation of a low cellular salt concentration. The nature of "apparent free space" is obscure, but for reasons given above, "apparent free spaces" cannot be simply a general osmotic compartment of the cell.

Cl regulation in *Tetrahymena* is qualitatively similar to Na regulation. Cl is maintained lower in the cells than in the environment. Cl_i appears to be constant (at least in adapted cells) in dilute media up to some Cl_o level, above which Cl_i increases linearly with increasing Cl_o. The saturation level of Cl exclusion is higher in adapted than in normal cells. These points suggest the possibility that the same mechanism is responsible for Na and Cl regulation. Na extrusion is not coupled with K accumulation in *Tetrahymena*, so Cl distribution may follow that of Na.

However, Na and Cl distributions are not associated in any simple manner. "Apparent free Cl space" is less than "Na space" in both adapted and normal cells. The rate of net Cl efflux from adapted cells after medium dilution is more rapid than net Na efflux. Cl_i increased whether Cl_o was increased by adding NaCl or KCl to the medium. Inorganic ions in *Tetrahymena* are not distributed according to a simple Donnan equilibrium. A separate mechanism for regulating Cl is possible, but this question and the relationship between Cl and cation distributions await further evidence.

In assessing ion distributions across cell boundaries, electrical potential gradients must be considered. Trans-surface potentials might be responsible for the differences in Na and Cl distributions. Direct measurements on *Tetrahymena* are not available. Trans-surface potentials and effects thereupon of varying the ionic environment have been measured in *Paramecium* (Kamada, 1934; Yamaguchi,

Table VI

Cellular potassium concentrations in some lower fresh-water invertebrates, determined by elemental analysis. (See Dunham and Child, 1961, Table I, for values from various sources determined by other methods)

Organism	K_i	Source
Acanthamoeba	27 meq./l. cells	Klein, 1959
Pelomyxa	34.5 meq./l. cell water	Riddle, 1962
Amoeba proteus	34 meq./kg. cells	Raab, unpublished
Tetrahymena (normal)	32 meq./l. cells	Dunham and Child, 1961
Tetrahymena (adapted)	33 meq./l. cells	Dunham, present report
Paramecium	30 meq./l. cells	Akita, 1941
Hydra littoralis	21, 30 meq./kg. wet weight	Steinbach, 1963
Chlorohydra	38, 50 meq./kg. wet weight	Steinbach, 1963
Dugesia	38 meq./kg. wet weight	Steinbach, 1962a,
Tubifex	27 meq./kg. wet weight	Dunham, unpublished
Anodonta (muscle)	10.5 meq./kg. wet weight 24.1 meq./kg. wet weight	Hayes and Pelluet, 1947 Florkin and Duchateau, 1950
Chlamydomonas	20 meq./l. cells	Ronkin and Buretz, 1960
Naegleria	24 meq./kg. cells	Dunham, unpublished

1960) and in an amoeba (Riddle, 1962). Unfortunately, reasonable extrapolations cannot be made from these studies to trans-surface potentials under most of the conditions employed in the present study.

The intracellular concentration of K (in meq./l. cells) is nearly the same in normal and adapted cells. The amount of K per cell is less in adapted than in normal cells (50.7 μeq./10^8 cells in normal, 31.6 μeq./10^8 cell in adapted), whereas after passive changes in cell volume, the amount of K per cell remains constant and the concentration changes, in both normal and adapted cells. Therefore, an alteration in K regulation accompanied adaptation to high NaCl, with the result that K concentration is the same in the two cell types. This observation suggests a minimal cellular K concentration (*cf.* Steinbach, 1962b). It is interesting in this regard to note the similarity of cellular K concentrations in a number of lower fresh-water invertebrates, listed in Table VI. This similarity among animals of some relatively unrelated groups is further indication of a minimal protoplasmic K concentration. Energetically, it is advantageous for cells of these animals to be as nearly isosmotic with the environment as possible (for a theoretical treatment, see Potts, 1954). However, there is a level of potassium below which cells cannot function. The common minimal K concentration to which representatives of several groups have evolved independently suggests that potassium is an inherent, integral protoplasmic constituent with a definite functional role.

SUMMARY

1. A culture of *Tetrahymena pyriformis* adapted to a high NaCl medium, containing 220 m*M* NaCl, was investigated. The concentration of NaCl in the medium of the normal (unadapted) animals was about 35 m*M*.

2. Upon direct transfer to the high NaCl medium, only 2% of the normal cells were sufficiently tolerant of the stress to survive and divide. Data were presented indicating that stress tolerance is a heritable character, and that this character was selected for upon transfer to the high NaCl medium.

3. The cell volume of the adapted cells was 45% less than that of the normal animals. The adapted cells were more nearly spherical than normal cells. Despite the smaller size of the adapted cells, the amount of non-volatile material per cell was the same in normal and adapted cells.

4. The main feature of the adaptation was a greatly increased ability of adapted cells to maintain a low cellular salt concentration. Sodium concentration in normal cells in normal medium was 13 meq./l. of cells. Sodium concentration in normal cells equilibrated in high NaCl medium was 105 meq./l. of cells. Sodium concentration in adapted animals was 43 meq./l. of cells two weeks after starting the culture, and fell gradually to 21 meq./l. of cells in 1500 generations (22 months). This constituted selection for ability to regulate sodium.

5. Two major differences in sodium regulation between normal and adapted cells were observed. First, the saturation level of the sodium extrusion mechanism, 20 m*M* in normal cells, increased to 120 m*M* in the adapted cells. Secondly, the "apparent free spaces" of both sodium and chloride were lower in adapted than in normal cells. The increased ability to regulate cellular sodium in adapted cells was held not to be due to a decrease in permeability to sodium.

6. From experiments utilizing an isotopic tracer, Na^{24}, 70% of cellular sodium was shown to be readily exchangeable with external sodium.

7. Potassium regulation was altered with adaptation such that potassium concentration per unit volume was the same in normal and adapted cells. The striking similarity of cellular potassium concentrations in *Tetrahymena* and a variety of other lower fresh-water invertebrates was pointed out. These points were discussed with respect to a general minimum protoplasmic potassium concentration.

LITERATURE CITED

AKITA, Y. K., 1941. Electrolytes in *Paramecium*. *Mem. Fac. Sci. Agric., Taihoku Imp. Univ.*, 23: 99–120.

ANDERSEN, B., AND H. H. USSING, 1960. Active transport. *In:* Comparative Biochemistry, vol. II, M. Florkin and H. S. Mason, eds., Academic Press, New York, pp. 371–402.

ANDRUS, W. D., AND A. C. GIESE, 1963. Mechanisms of sodium and potassium regulation in *Tetrahymena pyriformis* Strain W. *J. Cell Comp. Physiol.*, 61: 17–30.

BROWN, F., AND W. D. STEIN, 1960. Balance of water, electrolytes, and non-electrolytes. *In:* Comparative Biochemistry, vol. II, M. Florkin and H. S. Mason, eds., Academic Press, New York, pp. 403–470.

Bullock, T. H., 1960. Homeostatic mechanisms in marine organisms. *In:* Perspectives in Marine Biology, A. A. Buzzati-Traverso, ed., Univ. of California Press, Berkeley, Calif., pp. 199–209.

Chatton, E., and L. Tellier, 1927. Sur les limites de retour à l'equilibre physiologique de *Glaucoma pyriformis* porté dans les solutions hypertoniques de NaCl. *C. R. Soc. Biol.*, **97**: 780–784.

Crockett, R., P. B. Dunham and L. Rasmussen, 1964. Protein metabolism in *Tetrahymena pyriformis* cells dividing synchronously under starvation conditions. *C. R. Lab. Carlsberg,* **34**: in press.

Dunham, P. B., 1961. The physiological basis of acclimation of *Tetrahymena* to high NaCl medium. *Biol. Bull.,* **121**: 371.

Dunham, P. B., 1962. The adaptation of *Tetrahymena* to a high NaCl environment. *Biol. Bull.,* **123**: 462–463.

Dunham, P. B., and F. M. Child, 1961. Ion regulation in *Tetrahymena. Biol. Bull.,* **121**: 129–140.

Emerson, A. E., 1949. Ecology and evolution. *In:* Principles of Animal Ecology, W. C. Allee, A. E. Emerson, O. Park, T. Park and K. P. Schmidt, authors, W. B. Saunders Co., Philadelphia, pp. 598–729.

Florkin, M., and G. Duchateau, 1950. Concentrations cellulaire et plasmatique du potassium, du calcium, et du magnesium chez *Anodonta cygnea. C. R. Séances Soc. Belg. Biol.,* **114**: 1131–1132.

Gause, G. F., 1941. The effects of natural selection in the acclimatization of *Euplotes* to different salinities of the medium. *J. Exp. Biol.,* **87**: 85–100.

Gause, G. F., 1942. The relation of adaptability to adaptation. *Quart. Rev. Biol.,* **17**: 99–114.

Gordon, M. S., K. Schmidt-Nielsen and H. M. Kelley, 1961. Osmotic regulation in the crab-eating frog (*Rana cancrivora*). *J. Exp. Biol.,* **38**: 659–678.

Gross, W. J., 1961. Osmotic tolerance and regulation in crabs from a hypersaline lagoon. *Biol. Bull.,* **121**: 290–301.

Hamburger, K., and E. Zeuthen, 1960. Some characteristics of growth in normal and synchronized populations of *Tetrahymena pyriformis. C. R. Trav. Lab., Carlsberg,* **32**: 1–18.

Hayes, F. R., and D. Pelluet, 1947. Electrolytes in mollusc blood and muscle. *J. Mar. Biol. Assoc.,* **26**: 339–409.

Kamada, T., 1934. Some observations on potential differences across the ectoplasm membrane of *Paramecium. J. Exp. Biol.,* **11**: 94–102.

Keys, A., 1933. The mechanism of adaptation to varying salinity in the common eel and the general problem of osmotic regulation in fishes. *Proc. Roy. Soc. London, Ser. B,* **112**: 184–199.

Kidder, G. W., and V. C. Dewey, 1945. Studies on the biochemistry of *Tetrahymena.* III. Strain differences. *Physiol. Zoöl.,* **18**: 136–157.

Kidder, G. W., V. C. Dewey and R. E. Parks, 1951. Studies on the inorganic requirements of *Tetrahymena. Physiol. Zoöl.,* **24**: 69–75.

Kinne, O., 1958. Adaptation to salinity variations—some facts and problems. *In:* Physiological Adaptation. C. L. Prosser, ed., American Physiological Society, Washington, D. C., pp. 92–106.

Klein, R. L., 1959. Transmembrane flux of K^{42} in *Acanthamoeba. J. Cell. Comp. Physiol.,* **53**: 241–258.

Krogh, A., 1939. Osmotic Regulation in Aquatic Animals. Cambridge University Press, Cambridge, England.

Lerner, I. M., 1958. The Genetic Basis of Selection. John Wiley & Sons, New York.

Loefer, J. B., 1939. Acclimatization of fresh water ciliates and flagellates to media of higher osmotic pressure. *Physiol. Zoöl.,* **12**: 161–172.

Mast, S. O., and D. L. Hopkins, 1941. Regulation of the water content of *Amoeba mira* and adaptation to changes in the osmotic concentration of the surrounding medium. *J. Cell. Comp. Physiol.,* **17**: 31–48.

Potts, W. T. W., 1954. The energetics of osmotic regulation in brackish and fresh-water animals. *J. Exp. Biol.,* **31**: 618–630.

Prosser, C. L., 1955. Physiological variation in animals. *Biol. Rev.*, **30**: 229–262.

Prosser, C. L., 1958. The nature of physiological adaptation. *In:* Physiological Adaptation. C. L. Prosser, ed., American Physiological Society, Washington, D. C., pp. 167–180.

Riddle, J., 1962. Studies on the membrane potential of *Pelomyxa carolinensis*. *Exp. Cell Res.*, **26**: 158–167.

Ronkin, R. R., and K. M. Buretz, 1960. Sodium and potassium in normal and paralyzed *Chlamydomonas*. *J. Protozool.*, **7**: 109–114.

Scherbaum, O., 1957. The content and composition of nucleic acids in normal and synchronously dividing mass cultures of *Tetrahymena pyriformis*. *Exp. Cell Res.*, **13**: 24–30.

Scherbaum, O., and E. Zeuthen, 1955. Temperature induced synchronous divisions in the ciliate protozoan *Tetrahymena pyriformis* growing in synthetic and proteose-peptone media. *Exp. Cell Res., Suppl.*, **3**: 312–325.

Simpson, G. G., 1953. The Major Features of Evolution. Columbia University Press, New York.

Steinbach, H. B., 1962a. Ionic and water balance of planarians. *Biol. Bull.*, **122**: 310-319.

Steinbach, H. B., 1962b. The prevalence of K. *Perspectives in Biol. Med.*, **5**: 338–355.

Steinbach, H. B., 1963. Sodium, potassium, and chloride in selected hydroids. *Biol. Bull.*, **124**: 322–336.

Wigglesworth, V. B., 1938. The regulation of osmotic pressure and chloride concentration in the haemolymph of mosquito larvae. *J. Exp. Biol.*, **15**: 235–247.

Yamaguchi, T., 1960. Studies on the mode of ionic behavior across the ectoplasmic membrane of *Paramecium*. I. Electric potential differences measured by the intracellular microelectrode. *J. Fac. Sci. Univ. Tokyo, Sec. IV, Zool.*, **8**: 573-591.

Zeuthen, E., 1963. The temperature-induced division synchrony in *Tetrahymena*. *In:* Synchrony in Cell Division and Growth, E. Zeuthen, ed., John Wiley and Sons, New York. In Press.

MARINE
LIBRARY
MASS.
TORY

NUTRIENT TRANSPORT IN STARFISH. II. UPTAKE OF NUTRIENTS BY ISOLATED ORGANS[1]

JOHN CARRUTHERS FERGUSON

Cornell University,[2] Ithaca, New York

Evidence has been presented in a previous paper (Ferguson, 1964) that the coelomic fluid of starfish (*Asterias forbesi*) contains small but significant concentrations of amino acids and other materials which appear to be important in the process of nutrient translocation. These materials could be mobilized from the storage depots of the digestive glands and rapidly circulated in the coelomic fluid to other areas by the ciliary mechanisms described by Irving (1924) and Budington (1942). In order to verify this hypothesis, it is necessary to show that (1) nutrient materials may be released from the storage tissues into the coelomic fluid, (2) nutrient materials may be extracted from the coelomic fluid by the tissues requiring them, and (3) these operations may occur at rates sufficient to satisfy the metabolic needs of the animals.

These processes, common to most animals, may be quite readily studied in starfish. Several of the major organs of the body—the digestive glands, cardiac stomach, gonads, and rectal caeca—can be easily excised and maintained for considerable periods in clean sea water, which in composition is very similar to coelomic fluid (Cole, 1940). The extended survival of the organs under such conditions attests to the relatively autonomous existence they must normally lead. If the coelomic fluid is the medium of nutrient, waste, and gaseous transport, these preparations should approximate the actual conditions in the animals. In the present investigation, preparations of this type were used to confirm the occurrence of the coelomic transport mechanism of starfish, and to examine the properties and capabilities of this mechanism in nutrient translocation.

MATERIALS AND METHODS

The animals used in this work were freshly collected specimens of *Asterias forbesi* from the Woods Hole region, maintained in the laboratory in tanks supplied with adequate quantities of running sea water. Organs were carefully excised, rinsed in filtered sea water, and then placed in an incubative medium—either filtered sea water or cell-free coelomic fluid, extracted and pooled from several animals. Usually, organs weighing about 0.5 to 0.7 gm. were selected to be placed

---

[1] This work represents a part of a dissertation submitted to Cornell University in partial fulfillment of the requirements for the Ph.D. degree, granted in June, 1963. I am greatly indebted to Dr. John M. Anderson for his interest in its progress, and for his advice and encouragement. Much of the research was performed during tenure of a NSF Summer Fellowship. Additional financial support was provided from NSF Grant No. G-20744 to Cornell University.

[2] Present address: Florida Presbyterian College, St. Petersburg, Florida.

TABLE I

Uptake of nutrients by isolated tissues in sea water

Tissue	Nutrient	Weight gm.	Time hrs.	Init. Act. soln. mμc.	Final Act. soln. mμc.	Act. Removed mμc.	Final Act. Tissue mμc.
Rectal caeca	glucose	0.060	6	8,030 ± 113	6,690 ± 94	1,340 ± 147	1,410 ± 16
Rectal caeca	glucose	0.065	6	9,030 ± 126	7,420 ± 104	1,510 ± 163	1,240 ± 20
Rectal caeca	glycine	0.026	6	14,300 ± 100	8,450 ± 83	5,850 ± 130	6,850 ± 43
Rectal caeca	glycine	0.018	6	12,650 ± 88	4,290 ± 42	8,360 ± 120	5,570 ± 31
Gonads	glucose	0.190	8	17,950 ± 172	5,510 ± 55	12,440 ± 181	10,700 ± 43
Gonads	glycine	0.134	8	14,200 ± 140	4,800 ± 47	9,400 ± 148	7,740 ± 35
Stomach	glucose	0.090	3	1,280 ± 13	890 ± 10	390 ± 16	290 ± 3
Stomach	glucose	0.113	3	1,070 ± 9	830 ± 8	240 ± 11	230 ± 2

The organs were placed in 10 ml. of sea water together with small amounts of C^{14}-labeled nutrients. Absorption of the tracers by the tissues is indicated both by the disappearance of activity from the medium (Act. removed) and by subsequent demonstration of the activity in the tissues themselves (Final Act. Tissue). Error terms are based on the standard deviation of the counting rate.

in 25 ml. of medium to which a C^{14}-labeled nutrient had been added. When only small amounts of tissue could be obtained, as in the studies with rectal caeca, gonads, and stomach, only 10 ml. of medium were used. No attempt was made to maintain sterile conditions or to control pH. Irving (1926) has shown that the survival of digestive glands from *Patiria* placed in sea water is markedly affected by hydrogen ion concentration, but that the tissues will tend to modify the pH to their own optimum. He reports that digestive glands survive under such conditions for up to two days.

TABLE II

Uptake of nutrients by digestive glands in sea water

Nutrient	Time hrs.	Init. Act. soln. cpm.	Final Act. soln. cpm.	Act. removed by organ cpm.
Glucose	9	1,841 ± 9	152 ± 1.5	1,689 ± 9
Glucose	9	1,951 ± 14	140 ± 1.4	1,810 ± 14
Glucose	9	1,876 ± 13	169 ± 4.0	1,707 ± 14
Glycine	9	3,280 ± 23	54 ± 1.0	3,226 ± 23
Glycine	9	3,462 ± 25	89 ± 1.1	3,373 ± 25
Glycine	9	3,432 ± 24	135 ± 4.3	3,297 ± 24
APH*	9	4,319 ± 25	344 ± 2.5	3,965 ± 25
APH*	9	4,216 ± 25	596 ± 3.0	3,620 ± 24

* Algal protein hydrolysate.

This table shows the effect produced by isolated digestive glands when placed individually in beakers containing 25 ml. of sea water and a small amount of C^{14}-labeled material. The error terms are based on the standard deviation of the counting rate.

TABLE III

Uptake of nutrients by digestive glands in coelomic fluid

Nutrient	Time hrs.	Init. Act. soln. cpm.	Final Act. soln. cpm.	Act. removed by organ cpm.
Glucose	7	765 ± 5	53 ± 2	714 ± 5
Glucose	9	718 ± 5	54 ± 3	644 ± 6
Glycine	9	2,080 ± 17	557 ± 4	1,523 ± 17
Glycine	9	2,100 ± 17	524 ± 4	1,576 ± 17
APH*	8	1,607 ± 13	231 ± 3	1,376 ± 13
APH*	9	1,918 ± 19	252 ± 4	1,666 ± 19

* Algal protein hydrolysate.

This table shows the effect produced by isolated digestive glands when placed individually in beakers containing 25 ml. of pooled, cell-free coelomic fluid and a small amount of C^{14}-labeled material. The error terms are based on the standard deviation of the counting rate.

The cultures were maintained at a constant temperature of 21° C. in a bath of running tap water. Air was gently bubbled through the medium to keep it circulating. Three kinds of tracers were used—(1) algal protein-C^{14} hydrolysate (essentially a mixture of amino acids with about 20% undetermined material), (2) glycine-1-C^{14}, and (3) D-glucose-C^{14} (U.L.). In most of the experiments about 2.5 microcuries (μc.) were used in 25-ml. portions of medium, representing a concentration of nutrients approximating levels observed in normal specimens. Samples of the media (0.25 ml.) were drawn at intervals, diluted with 0.5 ml. of distilled water, and plated in duplicate 0.25-ml. aliquots on stainless steel planchets, which had been ringed with a wax pencil and coated with a spreading agent. The samples were counted on a Nuclear Chicago gas flow counter equipped with a "Micromil" window and an automatic sample changer. The quantity of tracer accumulated in some of the tissues was assayed at the end of the experiments by previously described methods (Ferguson, 1964).

In other experiments, total nitrogen and free ammonia nitrogen was measured

TABLE IV

Digestive glands in sea water with inhibitor

Nutrient	Time hrs.	Weight gm.	Init. Act. soln. cpm.	Final Act. soln. cpm.	Difference cpm.
Glucose	9	0.360	1,480 ± 15	1,350 ± 13	80 ± 20
Glucose	9	0.320	1,650 ± 15	1,520 ± 14	130 ± 21
Glycine	9	0.395	627 ± 9	495 ± 8	132 ± 12
Glycine	9	0.365	653 ± 10	474 ± 8	179 ± 13
Glycine	7	0.870	1,460 ± 14	1,160 ± 12	300 ± 18
Glycine	7	0.920	1,380 ± 14	1,078 ± 11	302 ± 18

These experiments were similar to those seen in Table II except that 2×10^{-4} *M* sodium iodacetate was added to the medium. The values for the activity removed (Difference) would be even lower except for self-absorption effects that occur in the samples. The error terms are based on the standard deviation of the counting rate.

in preparations containing 10 ml. of medium. Analysis of 2-ml. samples drawn from these was achieved by methods previously described (Ferguson, 1964). Inhibition of metabolic activity in some organs was induced with medium containing 2×10^{-4} M sodium iodoacetate.

RESULTS

In these experiments the tissues remained in an apparently healthy condition for over 12 hours and frequently demonstrated spontaneous movements. All showed the ability to remove small amounts of labeled nutrients from the media (Tables I, II and III). This uptake was greatly affected by metabolic inhibition (Table IV). The inhibition was probably more complete than would be indicated by the table, for much of the slight apparent decrease in radioactivity of the medium was most likely due to the increasing self-absorption of the samples as organic materials accumulated in the medium during the experiments.

Absorption of glucose-C^{14}

These experiments showed little difference in the ability of the digestive glands to remove low concentrations of labeled glucose from either sea water (Fig. 1) or

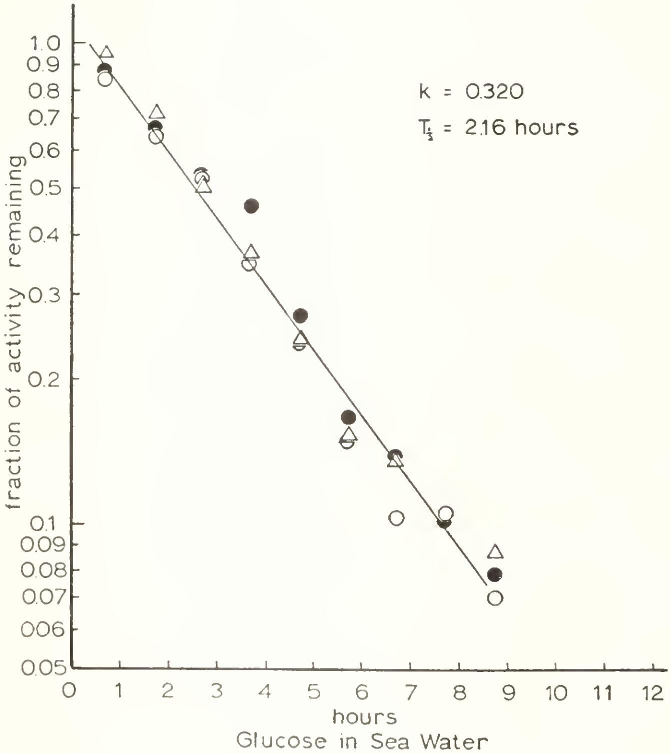


FIGURE 1. The rate of absorption of glucose-C^{14} from sea water by isolated digestive glands. The line represents the mean slope calculated from three experiments.

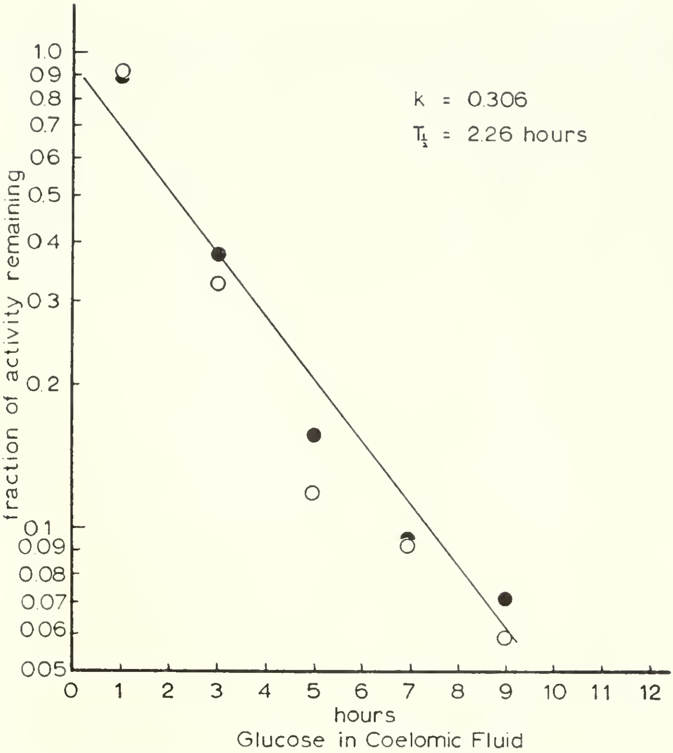


FIGURE 2. The rate of absorption of glucose-C^{14} from pooled, cell-free coelomic fluid by digestive glands in two experiments.

coelomic fluid (Fig. 2). In both cases the plotted data fitted a simple exponential function indicating that the rates of absorption were principally dependent on the concentrations of the tracer in the medium. The curves fitted to the data were calculated from the equation (Comar, 1955),

$$A = A_0 e^{-kt},$$

where A = the amount of activity present at time "t";
A_0 = the amount of activity present at zero time;
k = the constant representing the fractional rate of change of "A" with time.

The time required to remove one-half the tracer, the "half value time", ($T_{1/2}$), may be calculated from the expression,

$$T_{1/2} = -\frac{2.3 \log 1/2}{k} = \frac{0.693}{k}.$$

The half value time for the absorption of glucose-C^{14} by digestive glands in sea water was 2.16 hours; in coelomic fluid it was 2.26 hours.

The process being observed in this and the following experiments may not be just the simple removal of a substance from a fluid medium, but rather, it is more likely the turning-over of that substance—*i.e.*, for approximately every molecule of labeled material being removed from the fluid, another unlabeled molecule moves out to take its place. Some evidence that this is the case will be found in later data. If turning-over is occurring, the "turnover time" (T_t), the time required to exchange the number of molecules equivalent to the number present in the fluid at equilibrium, would be a very useful value.

Since k represents the fractional rate of change per unit time, its reciprocal would be the turnover time:

$$T_t = \frac{1}{k}.$$

Thus, the turnover time for the digestive glands placed in sea water with glucose-C^{14} would be 3.12 hours; in the coelomic fluid, 3.27 hours. As in the animals there are ten digestive glands active in about an equivalent amount of fluid, it may be estimated that the turnover time of glucose *in vivo* would be in the order of 0.33 hour.

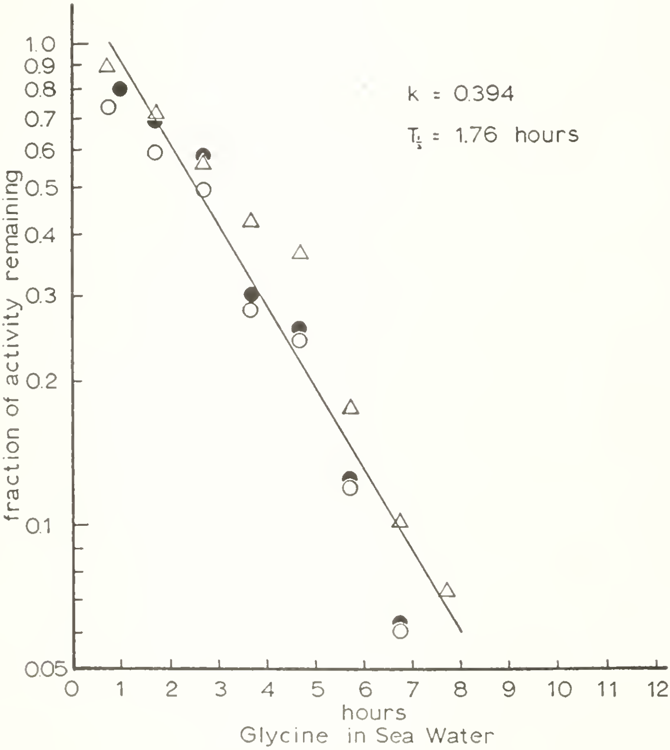


FIGURE 3. The rate of absorption of glycine-C^{14} from sea water by digestive glands in three experiments.

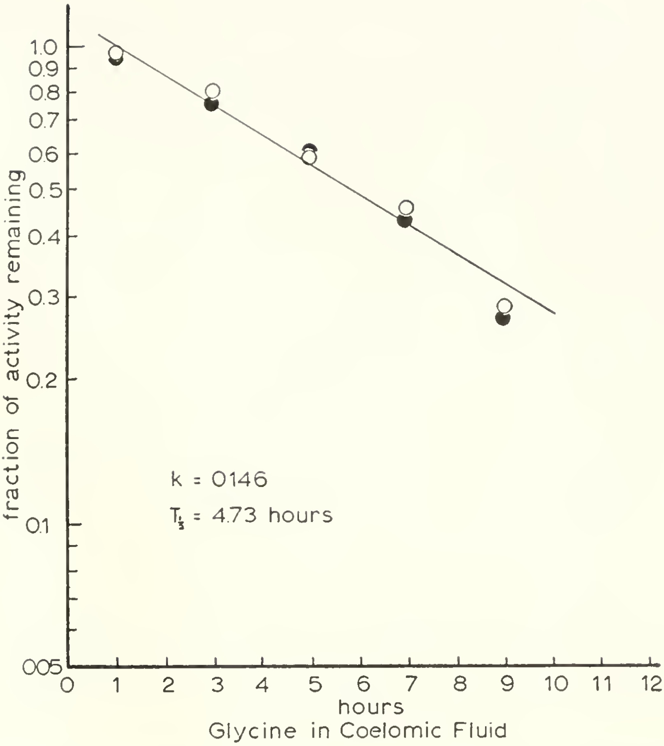


FIGURE 4. The rate of absorption of glycine-C^{14} from pooled, cell-free coelomic fluid by digestive glands in two experiments.

Absorption of glycine-C^{14}

The data obtained by incubating digestive glands with glycine-C^{14} were handled in the same manner as when glucose-C^{14} was used. With sea water as the medium, a $T_{1/2}$ of 1.76 hours was determined (Fig. 3). When the same experiment was performed in cell-free coelomic fluid, however, a much different value was obtained—a $T_{1/2}$ of 4.73 hours (Fig. 4). If the glycine that is being removed from the medium in these preparations is being replaced by unlabeled material diffusing out of the tissues, the turnover time in sea water would be 2.54 hours. In coelomic fluid it would be 6.85 hours, relating to a turnover time *in vivo* of approximately 0.69 hour.

Absorption of algal protein-C^{14} hydrolysate

Still different results were obtained when the digestive glands were incubated with algal protein-C^{14} hydrolysate (Figs. 5 and 6). There was a noticeably sharp bend at the five- or six-hour mark, correlating with the 20% of the mixture reported as "unidentified fractions" (probably less easily absorbed, higher molecular weight compounds). The slopes were calculated from the data taken before this

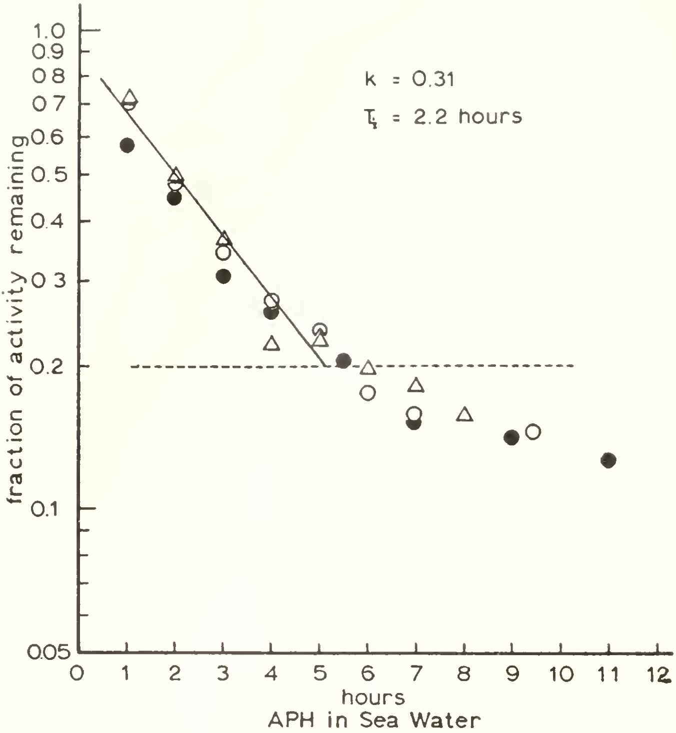


FIGURE 5. The rate of absorption of algal protein-C^{14} hydrolysate from sea water by digestive glands. The mean slope for the three experiments has been calculated only for the disappearance of the first 80% (dashed line) of the material.

point. There was little difference between the values obtained for the two media. Sea water produced a $T_{1/2}$ of 2.2 hours, while cell-free coelomic fluid gave a $T_{1/2}$ of 1.9 hours.

Again, if it is assumed that the amino acids being removed from the hydrolysate are being replaced by non-radioactive amino acids diffusing out of the digestive glands, the time required for turnover may be calculated. The turnover time in sea water turns out to be 3.22 hours. In coelomic fluid it is 2.70 hours. It is suggested, then, that an average value for the turnover of amino acids through the coelomic fluid of the starfish is approximately 0.27 hour.

Release of nitrogenous substances from digestive glands

Since the preceding experiments failed to show clearly that nutrients are given up to the coelomic fluid by the tissues, experiments were conducted to measure the release of nitrogenous materials from digestive glands placed in sea water. Figure 7 shows the appearance of total nitrogen in two such preparations. It could be expected from the experiments already presented that if nutrients were actually being given off and accumulated in the medium, they would tend to be reabsorbed at rates dependent on their concentrations. Likewise, since the digestive glands must represent such a large reservoir of nutrients, these substances should be released at rather constant rates. These ideas may be expressed mathematically in the equation,

$$\frac{dC_t}{dt} = \lambda - kC_t$$

where λ = the rate of nutrient loss from the tissue;
k = the reabsorption rate constant;
C_t = the concentration in the medium at time "t."

The equation is separable, and by integration a working form may be obtained:

$$C_t = \frac{\lambda}{k} + \left(C_0 - \frac{\lambda}{k} \right) e^{-kt},$$

where C_0 = the initial concentration in the medium.

Using this equation, the rate of nutrient loss and the fractional rate of reabsorption have been calculated from the data, and the curves obtained by using

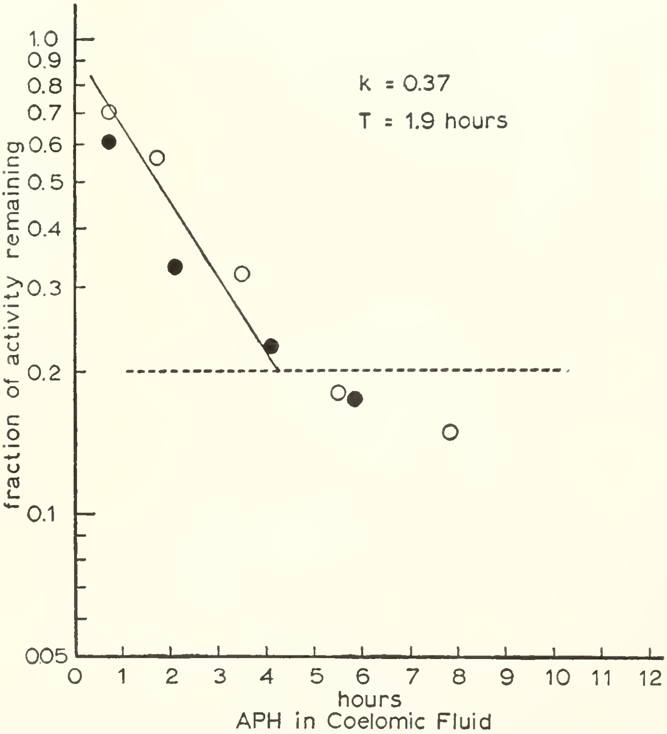


FIGURE 6. The rate of absorption of algal protein-C^{14} hydrolysate from pooled, cell-free coelomic fluid by digestive glands. The mean slope for the two experiments is from the first 80% of the activity removed.

these constants plotted in Figure 7. The concentrations expected at equilibrium would be 38.3 and 38.5 μg. N per ml. of medium. This is within the range that has been observed to occur in the animals (Ferguson, 1964).

As some of the total nitrogen observed in the preceding experiments must represent non-nutritional free ammonia released from the metabolizing tissues,

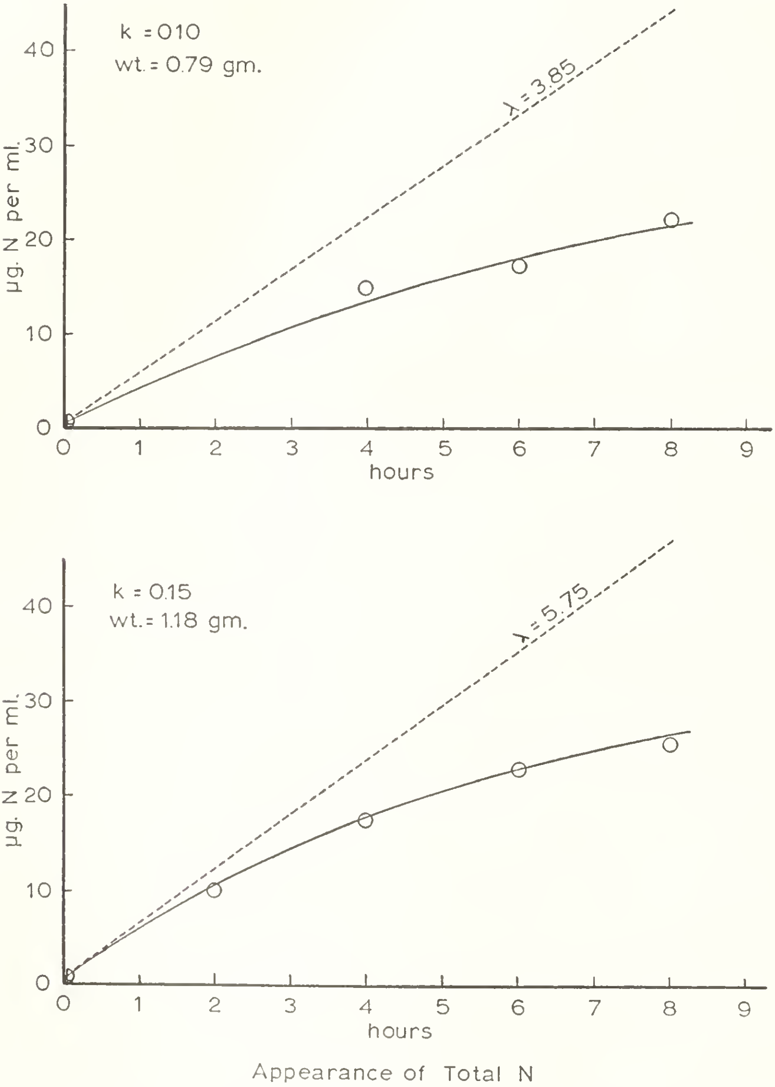


FIGURE 7. The rate of appearance in two experiments of nitrogenous substances in 10 ml. sea water containing pairs of digestive glands. The solid lines are the curves calculated to fit the data, and the dashed lines the estimated diffusion of material out of the digestive glands. The space between the solid and dashed lines represents material that has been reabsorbed by the tissues.

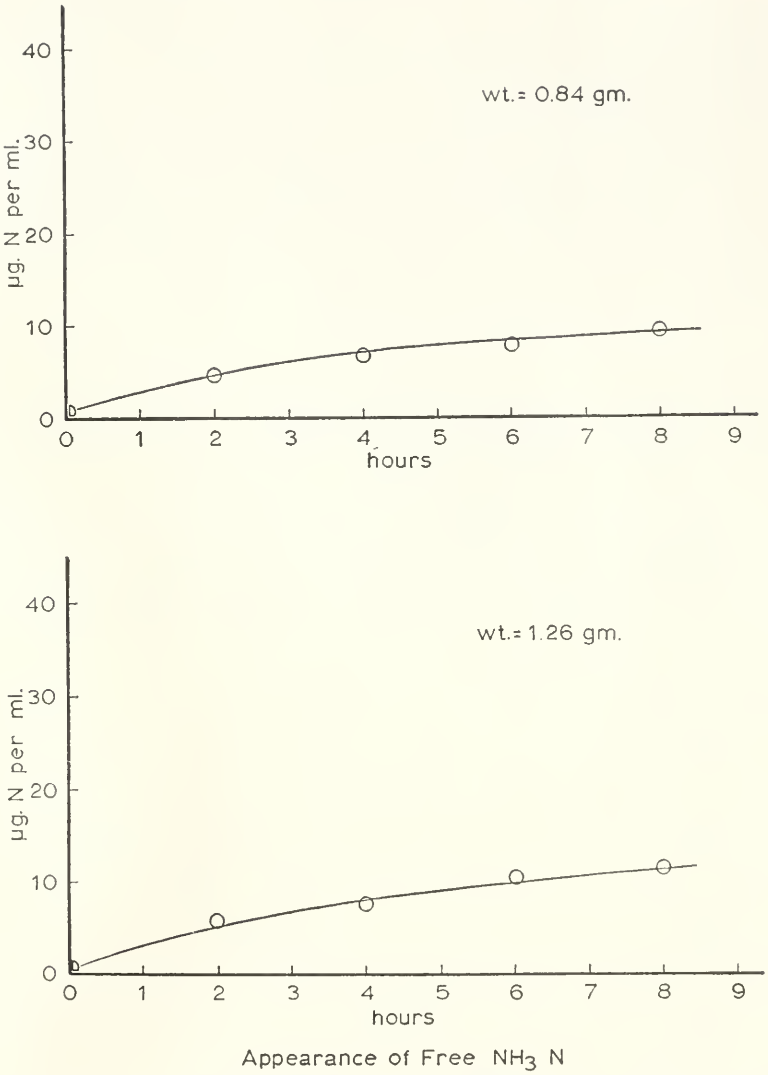


FIGURE 8. Two experiments demonstrating the rate of appearance of free ammonia in 10 ml. sea water containing pairs of digestive glands. The lines are subjective estimations of the curves.

similar experiments were performed to measure this factor. The results of two of these may be seen in Figure 8. The free ammonia values were subtracted from the comparable values of total nitrogen to give a measure of the movement of non-ammonia nitrogen into the medium. The estimates of non-ammonia nitrogen were treated in the same manner as used for the total nitrogen data. In the first case, for digestive glands weighing 0.79–0.95 gm., k was calculated as 0.25 and λ

as 3.5 μg. N per ml. per hour. In the second case, for digestive glands weighing 1.18–1.23 gm., k was 0.20 and λ, 3.6. Equilibrium levels for the two cases would occur at 18 and 14 μg. N per ml. fluid.

One further calculation is useful. The constant k, which represents the fraction of material removed per unit of time, can again be used to estimate the turnover time. For the non-ammonia nitrogen data, the turnover times of the two cases turn out to be 4.0 and 5.0 hours. As each of these experiments involved a pair of digestive glands, or one-fifth the total complement, the data would suggest that in the animal the time required to exchange all the nitrogenous nutrients in the coelomic fluid would be one-fifth of this, or, 0.8 to 1.0 hour.

Release of nitrogenous substances from inhibited digestive glands

It has already been shown (Table IV) that the absorption of nutrients by the tissues of the starfish is probably an active process. To further confirm this, preparations containing pairs of digestive glands in sea water with 2×10^{-4} M sodium iodoacetate were sampled and the concentrations of total nitrogen determined. The results of two such experiments are presented in Figure 9. As expected, reabsorption was inhibited and the full movement of material into the medium could be observed. The rates of release were fairly constant—7.9 gm. N per ml. per hour for the first set of glands (wt. 1.35 gm.) and 6.3 for the second

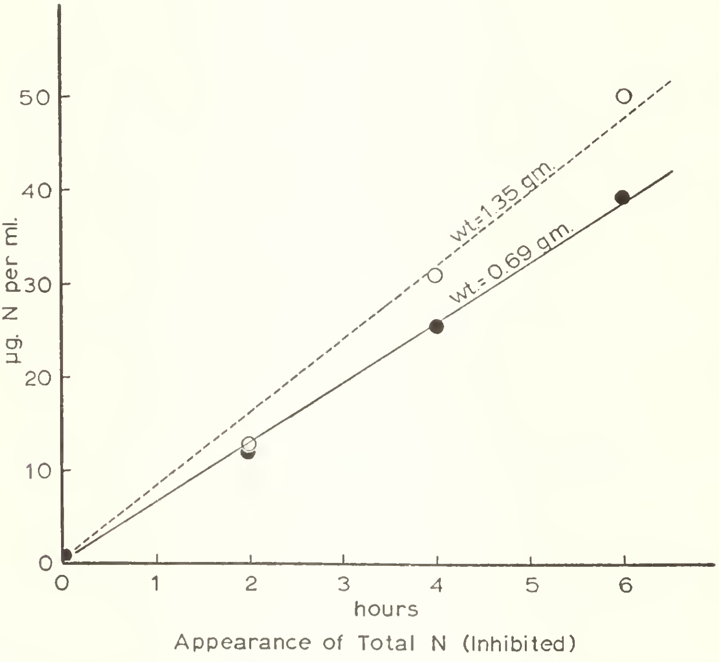


FIGURE 9. The rate of release of nitrogenous substances from pairs of digestive glands placed in 10 ml. sea water containing 2×10^{-4} M sodium iodoacetate. The lines are the mean slopes of the two experiments.

(wt. 0.69 gm.). These values are slightly higher than those estimated from the uninhibited material, the difference possibly being due to necrosis developing in the preparations. These data, too, may be used to calculate turnover times for nitrogenous materials in the coelomic fluid of animals if the equilibrium levels (38.3 and 38.5 μg. N per ml.) in the uninhibited total nitrogen experiments are accepted. The turnover time would be 0.97 hour in the first case and 1.22 hours in the second.

DISCUSSION

As all the organs studied demonstrated an ability to remove labeled nutrients from solutions of approximately the same concentration as found in normal coelomic fluid, one may conclude that in the living animal the different organs and tissues are continually depleting the coelomic fluid of the nutritional substance dissolved in it. This being the case, it should be expected that the concentration of nutrients measured in the body fluids of normal animals would be very low (Ferguson, 1964). The nutrients present must come from somewhere, and the only possible source is the tissues themselves. Figures 7, 8 and 9 indicate that this is the case, at least for nitrogenous materials. Nutrients are released, apparently passively, from the same tissues that are actively reabsorbing them.

Thus, these results give evidence that the organs and tissues of starfish are in a continual state of flux, with each both actively absorbing nutrients from the coelomic fluid and passively releasing them back into the same fluid. Presumably the same mechanism could serve to translocate nutrients from one region of the animal to another. It would be only necessary to have storage organs, such as the digestive glands, release material slightly more rapidly than they reabsorb it, and for the other tissues to absorb nutrients more rapidly than they release them.

There can be little doubt that these processes are of great significance in the economy of the animals, but it must be shown that they occur at rates sufficient to satisfy the metabolic needs of the starfish. The rate experiments with digestive glands are most useful in this respect. Digestive glands were chosen for this work not only because they were easy to obtain, but also because they represent the main source of nutrients appearing in the coelomic fluid. They comprise a very large proportion of the organic material in the animals, and they had previously been demonstrated to behave in a manner similar to the other tissues.

It was first noticed from these studies that nutrients were absorbed at rates dependent mainly on their concentration in the medium. A similar phenomenon was observed with injection studies by Van der Heyde (1922) (see Ferguson, 1964). It may be explained on the physical basis that as the solution becomes more dilute, fewer molecules come into contact with the tissues, and thus their extraction becomes increasingly difficult.

The turnover times calculated for these experiments were extended to give an estimate of the turnover time in the animals—20 minutes for glucose, 16 for a general mixture of amino acids, and 41 for glycine. These estimates were based on the fact that for equivalent amounts of fluid, there are ten times as many digestive glands in the animals as were used in the experiments. There is, of course, a great deal of tissue in the coelomic cavity of starfish in addition to that represented by the digestive glands. These other tissues must also be contributing to the process, so that the actual turnover times of the different substances through the fluid would be significantly shorter than the calculated ones. But these values do have meaning in that they reflect the maximum rates at which nutrients could depart from the digestive glands to supply the needs of the other tissues.

A few simple calculations serve to show that these rates should be adequate to sustain the animals. An average starfish contains about 25 ml. of coelomic fluid with a mean amino acid concentration of probably 30 μg. of nitrogen per ml. (Ferguson, 1964). Thus 750 μg. of amino acid nitrogen could be transported from the digestive glands every 16 minutes. As there are 1440 minutes in a day, this would represent a loss of nearly 68 mg. of amino acid nitrogen in a 24-hour period. Assuming a standard nitrogen content of the amino acids of about 16%, the 68 mg. would represent almost 0.5 gm. (dry weight) of protein a day. This is an amount far greater than that which animals kept in the laboratory could be induced to ingest in several days.

A similar argument could be developed from the glucose data which would show a maximum transport capacity of about 12 mg. of glucose per day. This, also, would seem to be in excess of the quantity of this substance normally ingested.

So far, only the average rates obtained from the different classes of compounds have been discussed. It is evident from Figures 4 and 6 that the normal rates of reabsorption of similar substances need not be at all similar. Glycine-C^{14} is absorbed from the coelomic fluid much more slowly than the general group of amino acids present in the algal protein-C^{14} hydrolysate. Since the experiments demonstrate great differences in the rates of reabsorption of glycine-C^{14} from sea water, where it is the only significant amino acid, and from coelomic fluid, which contains many different amino acids, it would appear that competitive inhibition is the factor chiefly responsible, although other mechanisms cannot be ruled out. There may be in the animals certain substances, specific amino acids, sugars, *etc.*, which are turned over much more rapidly than others and which are, therefore, the most significant metabolites in the process of nutrient transport. Just what these compounds are is at present unknown, but their identification would be an important topic for future research.

Giordano, Harper and Filice (1950), using a bioassay method, observed that the coelomic fluid of the starfish, *Pisaster brevispinus*, contained only five amino acids in measurable quantities. The most important of these was glycine, making up 67.4% of the total amino nitrogen. Serine represented 5.1%, arginine 7.2%, phenylalanine 5.1%, and tryptophan 0.1%. It may be expected that the different amino acids of the starfish would diffuse into the coelomic fluid from the various body regions at rates somewhat proportional to their concentrations in the tissues. Certainly the tissues of these animals must contain more amino acids than these five. Possibly the amino acids that are actually observed in the fluid in the greatest quantity are those that are the least readily reabsorbed by the tissues. It is seen that glycine represents the most abundant amino acid in a starfish and is also found to be much more slowly absorbed *in vitro* than the algal protein-C^{14} hydrolysate amino acids. By this same hypothesis, it is predictable that serine, and to a lesser extent, arginine and phenylalanine, would show signs of being less rapidly reabsorbed by the tissues. Experiments to verify this have not yet been performed.

There is no reason why amino acids and simple sugars need be the only nutrients transported by the coelomic fluid of starfish, although these seem to be far the most important. Other compounds, proteins, polypeptides, *etc.*, are present in the fluid, albeit in fairly low concentrations. While the roles of these materials are unknown, they may be functioning in the same fashion as the amino acids, but moving at considerably slower rates.

The experiments measuring the appearance of nitrogen from isolated digestive glands seem to indicate that this is so. They show that the turnover time for all the nitrogenous substances together is several times the average for the algal protein-C^{14} hydrolysate amino acids. While this difference could possibly be due to experimental methods, it more likely reflects the fact that the total nitrogen turnover includes not only rapidly moving amino acids, but also more slowly absorbed, larger molecules, especially proteins. These could be materials that were simply sloughed off by the tissues and are awaiting disposal, or they could be special classes of molecules produced for very limited and specific purposes. The occurrence of this latter group of substances is partially supported by recent evidence of endocrine or neurosecretory activity in starfish (Unger, 1962).

A careful look at the absorption curves (Figs. 1 through 6) permits another interesting observation. The fractional absorption rate for many of the nutrients used did not remain exactly constant. There is a definite tendency in most of the experiments for the rate to be rather low in the first few hours, followed by varying periods, each represented by several points, of more rapid absorption. These fluctuations may be due to background variations in the counting procedure, or they may be real differences in absorption rates reflecting behavior also occurring in the animals. If the latter is the case, reabsorption of nutrients in the body would not appear to be a constant and uniformly regulated process. Concentrations of nutrients in the coelomic fluid could fluctuate widely in rather short periods of time. Such fluctuations have been observed in serial analyses of experimental animals (Ferguson, 1964).

It has been concluded that the coelomic fluid is the principal medium of nutrient transport in *Asterias.* The evidence that has led to this conclusion has been based mainly on the nature and behavior of the tissues rather than on the composition of the coelomic fluid. Thus, one might expect the same sort of nutrient movement and exchange between tissues regardless of whether they are located in the perivisceral coelom or in some other space. The fluid cavities of the water vascular system and the perihaemal system must serve similar roles and all are, no doubt, important in supplying materials to the more peripheral parts, such as the tube feet and radial nerve cords. It is highly probable that the interstitial fluids between the cells and connective tissue of the body wall and other structures serve the same purpose. There is some evidence that the connective tissue in these forms is adapted to a very unspecialized interstitial medium possibly not greatly different from the coelomic fluid (Ferguson, 1960). Further study is required to clarify the nature and function of these fluids.

SUMMARY

1. Isolated digestive glands, gonads, rectal caeca, and cardiac stomach from the starfish, *Asterias forbesi,* were shown to be able to absorb labeled amino acids and

glucose from dilute solutions in sea water and coelomic fluid. This absorption could be largely inhibited with $2 \times 10^{-4}\ M$ sodium iodoacetate.

2. Analyses of the rates of absorption of labeled nutrients by isolated digestive glands have led to the conclusion that these substances are very rapidly turned over through all the body fluids of the starfish. Calculations indicate that the maximum possible rate of movement of stored nutrients (amino acids) from the digestive glands to the other tissues is equivalent to nearly 0.5 gm. (dry weight) of protein per day. The maximum rate for glucose is about 12 mg. per day. These quantities probably exceed the amount of nutrients normally ingested by the animals.

3. Similar studies indicate that related compounds may differ markedly in their importance in nutrient transport. The absorption of glycine is much slower from coelomic fluid than from sea water. It is suggested that the concentrations of this amino acid build up in the body fluids because its reabsorption may be inhibited by other amino acids.

4. Studies measuring the passive diffusion of nitrogenous materials from isolated digestive glands have further verified that a rapid flux of organic nutrients occurs between the internal fluids and tissues of the starfish.

LITERATURE CITED

Budington, R. A., 1942. The ciliary transport-system of *Asterias forbesi*. *Biol. Bull.*, **83**: 438–450.

Cole, W. H., 1940. The compositions of fluids and sera of some marine animals and the sea water in which they live. *J. Gen. Physiol.*, **23**: 575–584.

Comar, C. L., 1955. Radioisotopes in Biology and Agriculture. McGraw-Hill Book Co., Inc., New York.

Ferguson, J. C., 1960. The nature of the connective tissue of the body wall, retractor harness and cardiac stomach of the starfish, *Asterias forbesi*. *Anat. Rec.*, **138**: 348.

Ferguson, J. C., 1964. Nutrient transport in starfish. I. Properties of the coelomic fluid. *Biol. Bull.*, **126**: 33–53.

Giordano, M. F., H. A. Harper and F. P. Filice, 1950. The amino acids of a starfish and a sea urchin (Asteroidea and Echinoidea). *Wasmann J. Biol.*, **8**: 129–132.

Irving, L., 1924. Ciliary currents in starfish. *J. Exp. Zool.*, **41**: 115–124.

Irving, L., 1926. Regulation of the hydrogen ion concentration and its relations to metabolism and respiration in starfish. *J. Gen. Physiol.*, **10**: 345–348.

Lasker, R., and A. C. Giese, 1954. Nutrition of the sea urchin, *Strongylocentrotus purpuratus*. *Biol. Bull.*, **106**: 328–340.

Unger, H., 1962. Experimentelle und histologische Untersuchungen über Wirkfaktoren aus Nervensystem von *Asterias* (*Marthasterias*) *glacialis* (Asteroidea; Echinodermata). *Zool. Jahrb. Physiol.*, **69**: 481–536.

Van der Heyde, H. C., 1922. On the Physiology of Digestion, Respiration and Excretion in Echinoderms. Academical Dissertation, Amsterdam.

MORPHOLOGICAL COLOR CHANGE IN THE HAWAIIAN GHOST CRAB, OCYPODE CERATOPHTHALMA (PALLAS)[1,2,3]

JONATHAN P. GREEN[4]

Hawaii Marine Laboratory, University of Hawaii, Honolulu, Hawaii 96822, and the Department of Zoology, University of Minnesota, Minneapolis, Minnesota 55455

The functional activities of crustacean chromatophores are manifested by two types of color change. The relatively rapid mobilization of the pigment, leading to its concentration or dispersal within the chromatophore, is referred to as physiological color change. This facet of chromatophore activity is contrasted with morphological color change, a more enduring modification resulting from the production or destruction of pigment and/or chromatophores.

The relation between the physiological color change, as mediated by various hormonal agents, and the mechanisms controlling morphological color changes is imperfectly understood. Experimental studies in the past have been concerned principally with the hormonal control of the transitory physiological color changes (see reviews by Brown, 1961, and Fingerman, 1963). Few investigators have given any attention to the formation or destruction of crustacean pigments as a result of prolonged stimulation.

Sumner (1940) credits Flemming (1882) with the first observation that background shade could influence the total color pattern of an animal. Typical background responses result in dispersion of dark pigments on dark backgrounds and concentration on light backgrounds. Light colored chromatophores respond in an opposite manner. Babak (1912) postulated a relationship between physiological and morphological color changes, based on the state of dispersion of pigments within the chromatophore. This relationship, subsequently called Babak's Law, stated that the maintenance of a pigment in a concentrated state within the chromatophore is correlated with a reduction in the quantity of that pigment. Conversely, pigment dispersion is associated with pigment production.

Morphological color changes have been suggested to occur in the Crustacea (Keeble and Gamble, 1904) but only two papers have offered any experimental evidence for this phenomenon. Brown (1934) investigated the morphological color changes of the prawn, *Palaemonetes vulgaris*, maintained on various backgrounds. He observed that the red and blue pigments were the most rapidly formed

---

[1] Contribution Number 205 of the Hawaii Marine Laboratory, University of Hawaii, Honolulu, Hawaii 96822.

[2] This work constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from the Department of Zoology, University of Minnesota, Minneapolis, Minnesota 55455.

[3] Supported by a predoctoral research fellowship from the National Cancer Institute, U. S. P. H. S. (CF-9853).

[4] Present address: Department of Biology, Brown University, Providence, Rhode Island 02912.

and destroyed, while the yellow and white pigments were less reactive when the prawns were maintained on the appropriate background. Brown (p. 379) concludes that ". . . it is possible that pigment formation and destruction in adaptation to colored backgrounds may be controlled by the same humoral agents that are responsible for the control of migration of the pigments within the chromatophores."

Bowman (1942) counted crayfish (*Cambarus clarkii*) chromatophores and found that after 52 days on a black background the number of white chromatophores decreased as compared to the original number and that the number of red chromatophores increased. On a white background the converse was true.

Morphological color changes can be accomplished by more than one process: (1) by an increase (decrease) in the number of chromatophores per unit area of body surface (Bowman, 1942); (2) by an increase (decrease) in pigment concentration (Brown, 1934; Green, 1963); or (3) by a combination of the above.

The present study deals with the capacity of the Hawaiian ghost crab, *Ocypode ceratophthalma* (Pallas) to undergo morphological color change.

Materials and Methods

Male ghost crabs were collected periodically from four different areas on the islands of Oahu and Hawaii, Hawaii. The beaches on Oahu are composed of finely divided carboniferous, diatomaceous, and foraminiferous sand, and present a white background. In contrast to the Oahu beaches, the beach on Hawaii is composed of lava sand and presents a black background. The crabs from both beaches merit the name "ghost crab" because of their ability to blend with the background and their near invisibility when in motion.

Crabs were collected as needed on the Oahu beaches and placed in a bucket with sand. The time elapsed between collection and subsequent return to the laboratory seldom exceeded one hour. Crabs from the island of Hawaii were collected on a Thursday afternoon and placed by twos in wax-coated paper cups with black sand on the bottom and maintained in these containers until the following Tuesday afternoon.

Upon reaching the laboratory the crabs were washed free of adhering sand and the number of black chromatophores on the anterior surface of the meropodite of the fourth right walking leg counted with the aid of a Bausch and Lomb dissecting microscope (× 13). After the chromatophores were counted the approximate area of the anterior surface of the meropodite was determined by direct measurement. Analysis of the number of black chromatophores per unit area of the legs of the crabs from the three Oahu beaches employed as collection sites indicated that the crabs were comparable with respect to this parameter.

The animals were then placed on one of four regimes:

A. White background—constant illumination
B. Black background—constant illumination
C. Total darkness
D. White background—intermittent illumination (12 hours light, 12 hours dark).

The animals were kept in individual glass vessels three inches high and three inches in diameter, painted either white or black on the outside. One-fourth inch

TABLE I

Effect of various regimes on the black chromatophore index

Collection location	Number of animals	Treatment	Time on treatment (days)	Black chromatophore index Mean ± S.E.
White sand beaches	160	—	0	3.14±0.10
White sand beaches	17	White background, constant illumination	7	4.60±0.65
White sand beaches	21	White background, constant illumination	14	5.15±0.71
White sand beaches	8	White background, constant illumination	21	5.18±0.76
White sand beaches	3	White background, constant illumination	28	5.48±2.09
White sand beaches	18	Black background constant illumination	7	5.32±0.49
White sand beaches	9	Black background, constant illumination	14	8.67±1.23
White sand beaches	4	Black background, constant illumination	21	6.35±1.34
White sand beaches	6	Total darkness	7	3.01±0.32
White sand beaches	5	Total darkness	14	3.13±0.30
White sand beaches	5	White background, intermittent illumination	7	3.07±0.58
White sand beaches	5	White background, intermittent illumination	14	2.71±0.51
Black sand beach	24	—	0	37.22±0.70
Black sand beach	5	White background, constant illumination	7	32.66±2.03

sea water was kept in each vessel and fresh sea water was added every two or three days. The animals were unfed during the experimental period (which amounted to at most four weeks for any one animal). Blanks in the data are due to death of the experimental animal. The animals were illuminated by General Electric "daylight" fluorescent light bulbs. The animals maintained in total darkness were kept in a photographic darkroom and were illuminated by a Nicholas illuminator only for the time necessary to count their chromatophores (about one minute). After 7, 14, 21, and 28 days had elapsed the numbers of black chromatophores on the same area of the leg were recounted. In order to facilitate counting chromatophores with dispersed pigment, *i.e.,* from animals from the black sand beach or those on black

TABLE II

The relationship between black chromatophore number and the area of the anterior surface of the meropodite of the fourth right walking leg of newly caught Ocypode ceratophthalma

Collection location	Number of animals	Number of black chromatophores		Leg area (mm.^{2})	
		Range	Mean ± S.E.	Range	Mean ± S.E.
White sand beaches	160	11–261	70 ± 4	6.0–51.5	21.0 ± 0.87
		Black chromatophore index = 3.14 ± 0.01			
Black sand beach	24	250–630	425 ± 23.5	6.4–16.8	11.4 ± 0.12
		Black chromatophore index = 37.22 ± 0.70			

backgrounds, the expanded chromatophores were made to contract by placing the animals in white bowls for two hours.

RESULTS

The data are presented in Table I. The means listed in the table are those of the black chromatophore number per unit area (mm.^{2}) of the anterior surface of the meropodite of the fourth right walking leg (black chromatophore index) ± the standard error of the mean. The relationship between black chromatophore number and the area of the meropodite of newly caught animals is presented in Table II.

Regression analyses have been obtained for the animals maintained on the various regimes. In all cases the black chromatophore index has been related to the length of time that the animals were maintained on the various backgrounds.

Figure 1A indicates that white sand animals maintained on white background with constant illumination did not show a significant regression between black chromatophore index and time of maintenance on the background. Further analysis reveals, however, that this curve contains a component which indicates a significant increase in black chromatophore number during the first seven days on the white background. Thereafter (7 through 28 days) no further significant change occurs.

Figure 1B indicates that there has been a highly significant increase in black chromatophore index (+ 0.3 black chromatophore per mm.^{2} per day) in white sand animals maintained on a black background. This increase is considered to be linear over the experimental period. The decrease observed between the 14th and 21st days is not statistically significant. The high variability of the mean for the 21st day is in part due to the small number of surviving animals.

Animals maintained in total darkness (Fig. 1C) and under intermittent illumination—white background (Fig. 1D) showed no significant variation in black chromatophore number during the experimental period.

The data for the black sand animals on white background have not been plotted because only two points are available (0 and 7 days). However, a comparison between the mean black chromatophore index for these two points indicates that there has been a significant decrease in black chromatophore index (− 0.76 black chromatophore per mm.^{2} per day) over the experimental period.

During the experimental period the black chromatophores of those black sand animals kept on a white background underwent certain structural changes. The pigment within the black chromatophores contracted and the pigment granules became densely packed together at the cell center. Due to the large number of black chromatophores and their syncytial nature in the hypodermis lining the carapace, the animals did not immediately appear markedly lighter. Several days later, after being on the white background under constant illumination, some of the black chromatophores appeared to be degenerating. Dark-colored particles could be seen scattered among the intact chromatophores. In general, the freed pigment particles tended to form a corona about the old cell center. The processes of the black chromatophores of white sand animals kept on a black background showed increased arborization and processes of adjoining "expanded" chromatophores intermingled, thereby temporarily losing their identity.

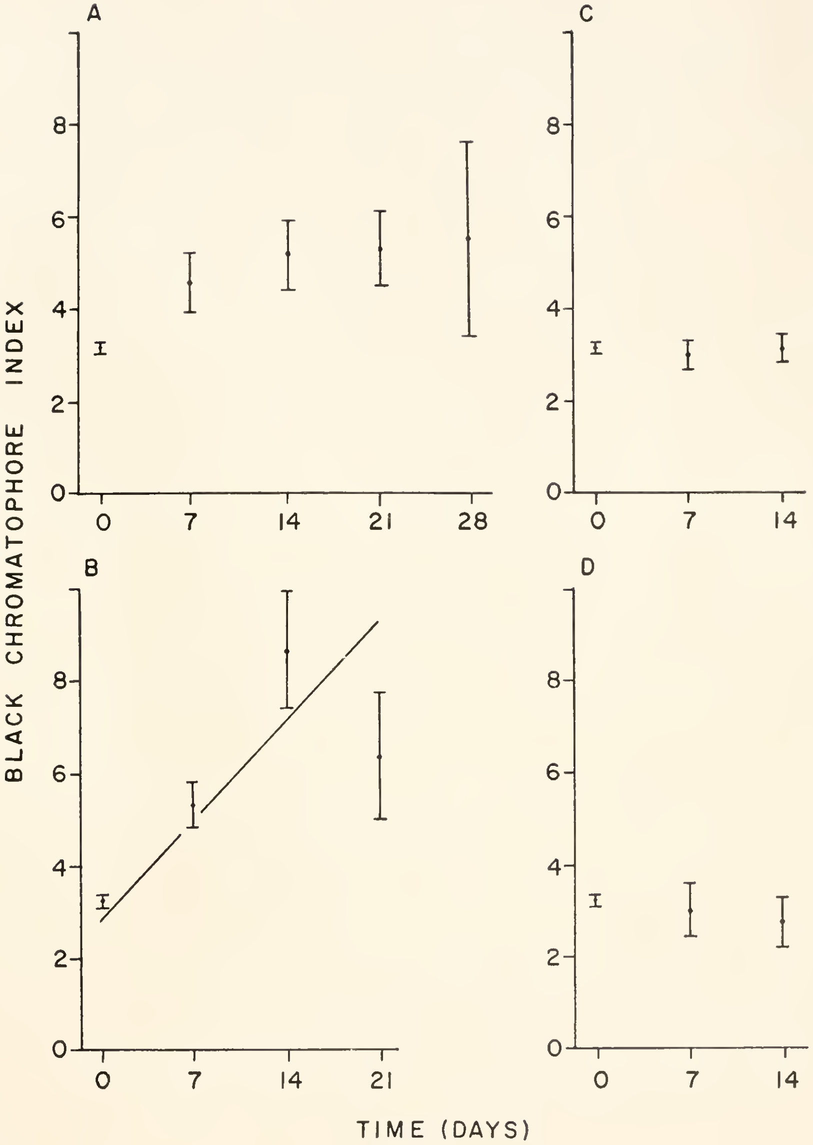


FIGURE 1. The relationship between black chromatophore index and time spent on background (for crabs of white sand history). The $P = 0.95$ confidence interval of the slope of the linear regression line is given. A. White background—constant illumination. $-0.005 \leqslant b \leqslant +0.249$. B. Black background—constant illumination. $+0.243 \leqslant b \leqslant +0.353$. C. Total darkness. $-0.110 \leqslant b \leqslant +0.050$. D. White background—intermittent illumination. $-0.075 \leqslant b \leqslant +0.067$.

DISCUSSION

The two populations of Hawaiian ghost crab, one from the white sand beaches of Oahu and the other from the black sand beach of Hawaii, are (aside from pigmentation) morphologically indistinguishable. The black sand crab has, however, nearly 12 times as many black chromatophores as does its white sand counterpart.

Long-term maintenance of crabs on particular backgrounds in some cases effects changes in the number of black chromatophores. The most interesting cases are those of white sand animals on black background and of black sand animals on white background. In the first case there has been a highly significant increase in the black chromatophore index and in the second case a highly significant decrease. This finding can be contrasted with crabs maintained in total darkness or under conditions of intermittent illumination (white background). Crabs in the latter two regimes did not show any significant variation in black chromatophore index over the same time period. The black chromatophores of white sand animals on white background increased during the first seven days on white background but thereafter remained at this new level for the remainder of the experimental period.

I am unable to explain the increase in the black chromatophore index of animals of white sand history maintained on a white background, but it is noteworthy that this increase persisted only for the first seven days and no further increase thereafter occurred. This group of crabs was under unnatural light conditions and therefore it is not particularly surprising to find differences between animals continuously illuminated and those which were intermittently illuminated.

We can conclude from these results that, in addition to other less well-known factors, illumination and background shade influence formation and destruction of chromatophores in *Ocypode*.

I would like to acknowledge the helpful suggestions and pertinent criticisms of my graduate advisor, Professor Grover C. Stephens. Dr. S. J. Townsley of the Department of Zoology, University of Hawaii, read and criticised an early draft of this paper and also verified my identification of the crabs.

SUMMARY

1. The black chromatophores of two populations of the Hawaiian ghost crab, *Ocypode ceratophthalma* (Pallas), were investigated with respect to the effect of long-term background adaptation.

2. Crabs from the black sand beach of Hawaii have twelve times the number of black chromatophores as do crabs from the white sand beaches of Oahu.

3. Crabs of white sand history maintained on a black background in the laboratory showed an increase of 0.3 black chromatophore per mm.^{2} per day over a 28-day period.

4. Crabs of black sand history maintained on a white background in the laboratory showed a decrease of 0.76 black chromatophore per mm.^{2} per day over a 7-day period.

5. The formation and destruction of black chromatophores in *Ocypode* is related to factors of illumination and background shade.

LITERATURE CITED

Babak, E., 1912. Über den Einfluss des Lichtes auf die Vermehrung des Hautchromatophoren. *Arch. ges. Physiol.*, **149**: 462–470.

Bowman, T. E., 1942. Morphological color change in the crayfish. *Amer. Nat.*, **76**: 332–336.

Brown, F. A., Jr., 1934. The chemical nature of the pigments and the transformations responsible for color changes in *Palaemonetes*. *Biol. Bull.*, **67**: 365–380.

Brown, F. A., Jr., 1961. Chromatophores and color change. *In:* Comparative Animal Physiology, C. L. Prosser and F. A. Brown, Jr., Editors, W. B. Saunders Co., Philadelphia, Chapter 19.

Fingerman, M., 1963. The Control of Chromatophores. Macmillan, New York.

Flemming, W., 1882. Kernsubstanz, Kern und Zelltheilung. Leipzig.

Green, J. P., 1963. An analysis of morphological color change in two species of brachyuran crustaceans. Ph.D. Thesis, University of Minnesota, Minneapolis.

Keeble, F., and F. W. Gamble, 1904. The colour physiology of higher crustacea. *Phil. Trans. Roy. Soc. London, Ser. B*, **196**: 295–388.

Sumner, F. B., 1940. Quantitative changes in pigmentation resulting from visual stimuli in fishes and amphibia. *Biol. Rev.*, **15**: 351–375.

STUDIES ON SHELL FORMATION. X. A STUDY OF THE PROTEINS OF THE EXTRAPALLIAL FLUID IN SOME MOLLUSCAN SPECIES[1,2]

SHINJIRO KOBAYASHI[3]

Department of Zoology, Duke University, Durham, North Carolina

The crystals and organic matrix of shell are formed within a thin layer termed the extrapallial fluid (de Waele, 1930) enclosed between the mantle and the inner shell surface. Accordingly, this fluid is considered to contain precursors of the organic matrix, calcium carbonate in the state just prior to its deposition, carbon dioxide, and other substances. At the same time, it is probable that physico-chemical characteristics and composition of the fluid will determine the nature of the organic matrix and type of calcium carbonate crystals deposited, that is, whether aragonite, calcite, or vaterite.

Recently evidence has accumulated on the relation of the organic matrix of shell to the shell layers, to taxonomic groups, and to the crystallization of calcium carbonate. For instance, the proteins contained in the prismatic and nacreous layers of a shell (Roche *et al.*, 1951; Hare, 1963) and also those in the prismatic and nacreous portions of cultured pearls (Yasuda *et al.*, 1957; Tanaka *et al.*, 1960) differ in the amino acid composition of the parts. Grégoire *et al.* (1955) and Grégoire (1957) have demonstrated a difference of the lace-like pattern of the organic matrix of the nacreous layer in the various groups of molluscs. Watabe and Wilbur (1960) have reported experiments suggesting that in the shell formation of molluscs the nature of the organic matrix has an important role in determining the crystal forms. Recently, Kitano and Hood (unpublished data) reported the effect of organic material upon the crystal forms of calcium carbonate *in vitro*.

While the importance of the extrapallial fluid in shell formation has been emphasized by de Waele (1930), Robertson (1941), Stolkowski (1951), and others, most of the previous investigations in this field have been concentrated upon the characteristics of the mantle and shell itself, omitting consideration of this fluid. Accordingly, our knowledge concerning the protein components of the extrapallial fluid is almost completely lacking. The present experiments were undertaken, as an initial study in this field, to detect the distribution of these proteins in some molluscan species by means of paper and cellulose acetate electrophoresis.

---

[1] Supported by grants from the Office of Naval Research and the National Institutes of Health.

[2] The writer wishes to express his thanks to Professors K. M. Wilbur and W. Rundles and to Dr. N. Watabe for their suggestions and for facilities which have been made available for the present work; he is also indebted to Mr. W. Ridenhour for his technical assistance.

[3] Present address: Faculty of Fisheries, Hokkaido University, Hakodate, Japan.

MATERIALS AND METHODS

The following fourteen species were used: (1) *Aequipecten irradians concentricus*, (2) *Chlamys nipponensis*, (3) *Crassostrea virginica*, (4) *C. gigas*, (5) *Ostrea edulis*, (6) *O. lurida*, (7) *Modiolus demissus*, (8) *Busycon carica*, (9) *Mercenaria mercenaria*, (10) *Elliptio complanatus*, (11) *Cristaria woodiana*, (12) *Corbicula leana*, (13) *Viviparus intertextus*, and (14) *Cipangopaludina malleata*. Of these, the first nine species are marine and the latter five fresh-water forms. It has been known that in the normal state, calcium carbonate crystals of the shell in the species of (1)–(6) are wholly composed of calcite, those of (7) of calcite in the prismatic layer and of aragonite in the nacreous layer, and those of (8)–(14) wholly aragonite (Bøggild, 1930; Bronn, 1935; Stolkowski, 1951; Lowenstam, 1954; Odum, 1957; Turekian and Armstrong, 1960; Wada, 1961).

Fluids of marine forms were usually taken from animals freshly collected at the Duke Marine Laboratory, Beaufort, North Carolina. In other cases, both marine and fresh-water animals were maintained in the Department of Zoology, Duke University. In the Japanese marine forms, fluids were collected from animals which had been cultured in hanging cages at a depth of about 1.5 meters in Hakodate Harbor, following a manner similar to the pearl-oyster culture; fresh-water forms were reared in tanks of a green-house of the Faculty of Fisheries, Hokkaido University.

For collection of the extrapallial fluid, the following method was simplest and most satisfactory. After opening the valves by means of a shell speculum used in pearl culture, a tiny hole was made by separating some pallial muscle from the inner surface of the shell. A long injection needle on a syringe was inserted through this hole into the extrapallial space, always being careful that the tip of the needle was in contact with the inner surface of the shell. The extrapallial fluid was withdrawn by gentle suction. In the case of *Busycon carica, Viviparus intertextus* and *Cipangopaludina malleata*, the fluid was collected in a similar manner by inserting a needle into the extrapallial space and withdrawing fluid after the foot had been extended. Usually, fluid collected from 2–10 specimens was pooled and used for each test. Blood was carefully collected by heart puncture and gentle suction applied at the time of each beat.

The extrapallial fluid was centrifuged and the supernatant was concentrated to about one-tenth its original volume by dialysis against 20% gum arabic for about 20–30 hours at 4° C. During dialysis, a whitish precipitate formed in the concentrated fluid. No distinct difference of the electrophoretic pattern was found between the runs on the supernatant only and the mixture of both fluid and precipitate. Blood was concentrated to about one-third its original amount by dialysis against 10% gum arabic at 4° C. for about 16–20 hours.

An E–C Apparatus Co. paper electrophoresis system was used. Most of the procedures were performed according to the recommendations made by Raymond (1955) for this apparatus. In most cases, six strips were run simultaneously, including a control of human serum. On the basis of preliminary experiments, the following conditions were chosen: barbital buffer solution (Holt *et al.*, 1952), pH 8.5 and ionic strength 0.045; delivered constant current intensity 0.8 mA/cm. width of the strip, and voltage-gradient during each run about 10–11 V/cm. length of the strip; cooling temperature of the apparatus 10° C.; duration of run 4.5 hours; Whatman 3MM filter paper strip of 3.1 × 46.5 cm.; a sample of 0.05–0.1 ml. was applied to the strip at its origin as a straight streak 1.5 cm. wide with a microsyringe. The amount of human serum applied was 0.01 ml. When a relatively high current intensity was delivered, a better electrophoretic pattern was obtained, possibly owing to high viscosity of the concentrated extrapallial fluid applied, though such a condition was not desirable for human serum used as a control. After completion of the electrophoresis, the strips were dried at about 100° C., rinsed in methanol for 10 minutes, and stained for 30 minutes in a 0.1% solution of bromphenol blue in methanol; after washing successively in three changes of 2% acetic acid, the strips were dried in air; the color was intensified by exposure to ammonia vapor and the strips were then scanned with a Spinco Model RA Analytrol.

The paper electrophoresis on the fluid of *Crassostrea gigas*, *Ostrea edulis*, *O. lurida*, *Chlamys nipponensis*, *Cristaria woodiana*, *Corbicula leana*, and *Cipangopaludina malleata* was conducted in Japan under the following conditions: barbital buffer solution, pH 8.5 and ionic strength 0.045; delivered constant current intensity 0.5 mA/cm. width of the strip, and voltage-gradient during each run about 10–12 V/cm. length of the strip; cooling temperature of the apparatus 15° C.; duration of run 3–3.5 hours; Toyo No. 51 filter paper 12.5 × 26 cm. (four simultaneously run); amount of sample 0.05–0.1 ml. A modified type of Grassman-Hannig's electrophoresis apparatus was used. Scanning of the strips was made with a Natsume densitometer after clearing them in melted paraffin.[4]

Electrophoresis on cellulose acetate was made under the following conditions in which most of the procedures were performed according to Smith (1960): Laurell's barbital buffer solution, pH 8.6, 0.07 M solution for impregnation of the strips and 0.06 M for electrophoretic run; strip size 2.5 × 18 cm., connected with two filter paper strips of 15 cm. length and 2.5 cm. width which were long enough to reach down into the buffer solution in tanks on both sides of the apparatus. Certain practical difficulties were encountered in the use of cellulose acetate strips. For instance, the sample was hardly absorbed on the strip when applied, owing to its high viscosity; it was not rare that it took one or two minutes to absorb. So, even if the sample was applied to the cellulose acetate strip so as to be linear at its origin, by use of the microsyringe, usually it spread around very slowly, forming a round spot. Accordingly, it was difficult to obtain a satisfactory electrophoretic pattern, especially with samples from species having complex protein fractions. In spite of such disadvantages, separation of the protein fractions was found to be better than that obtained from the paper electrophoresis.

RESULTS

The electrophoretic patterns of the extrapallial fluid for each species are presented in Figures 1 and 2. Generally, separation of the protein fractions by paper electrophoresis was not clear as in the case of the human serum, possibly owing to abundance of mucoidal substances. However, as is seen in Figure 1 B, the

---

[4] The writer wishes to thank Professor T. Imai of Tohoku University, Japan, for kindly supplying the specimens of *Ostrea edulis* and *O. lurida* which had been transported from Europe and cultured in his tanks, and also to thank Dr. Yuki and Messrs. K. Imamura, Y. Ise, and K. Shiota for their kind help in the experiments made in Japan.

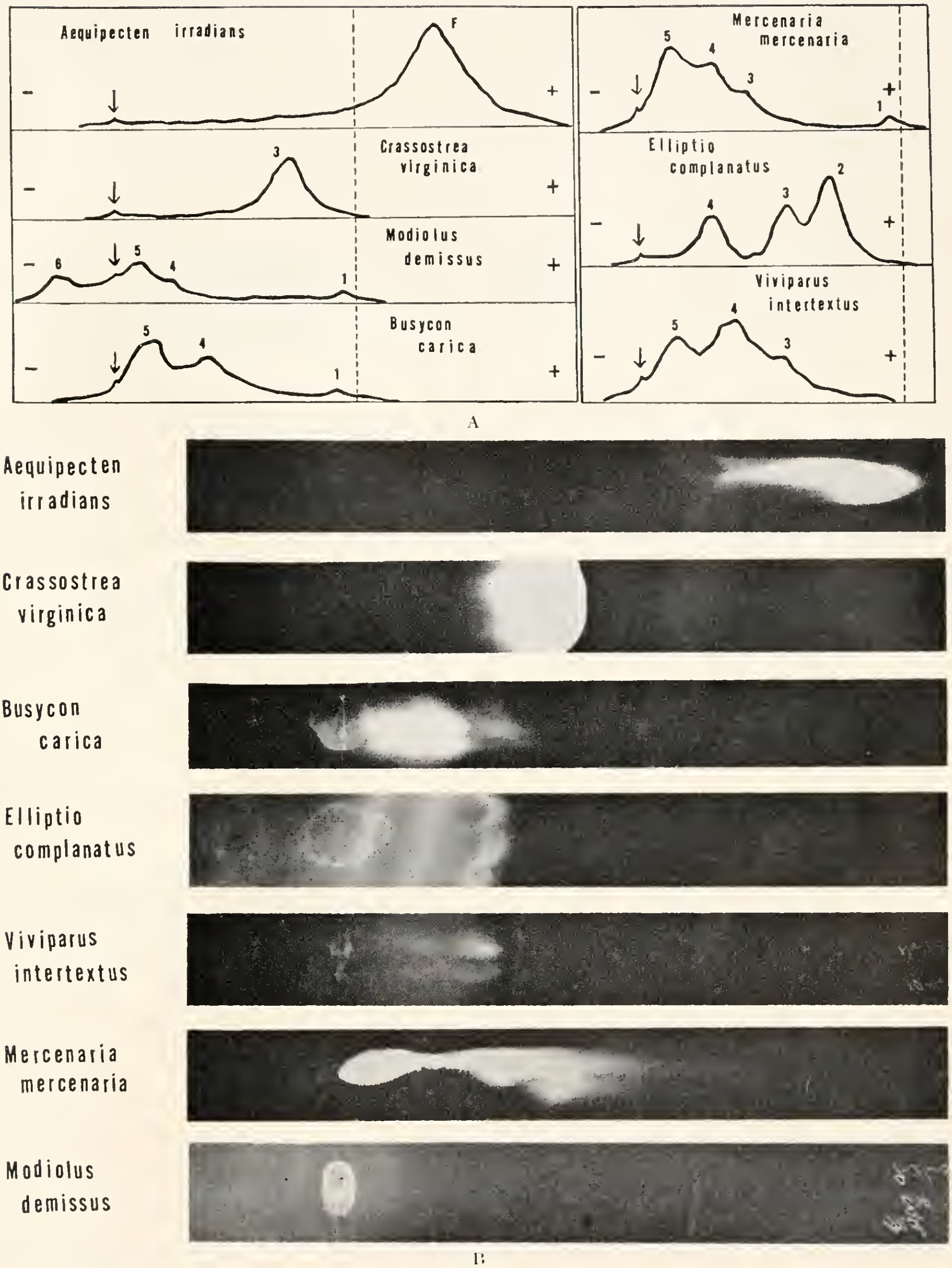


FIGURE 1. Electrophoretic patterns of the proteins of the extrapallial fluids in seven molluscan species. A: scannings obtained from paper electrophoresis; arrows indicate the origin of the strip; dotted line the relative migration distance of albumin of human serum. B: cellulose acetate electrophoretic diagrams of the proteins of the extrapallial fluids; all strips were stained with Ponceau S, but not cleared, and printed directly on photographic paper.

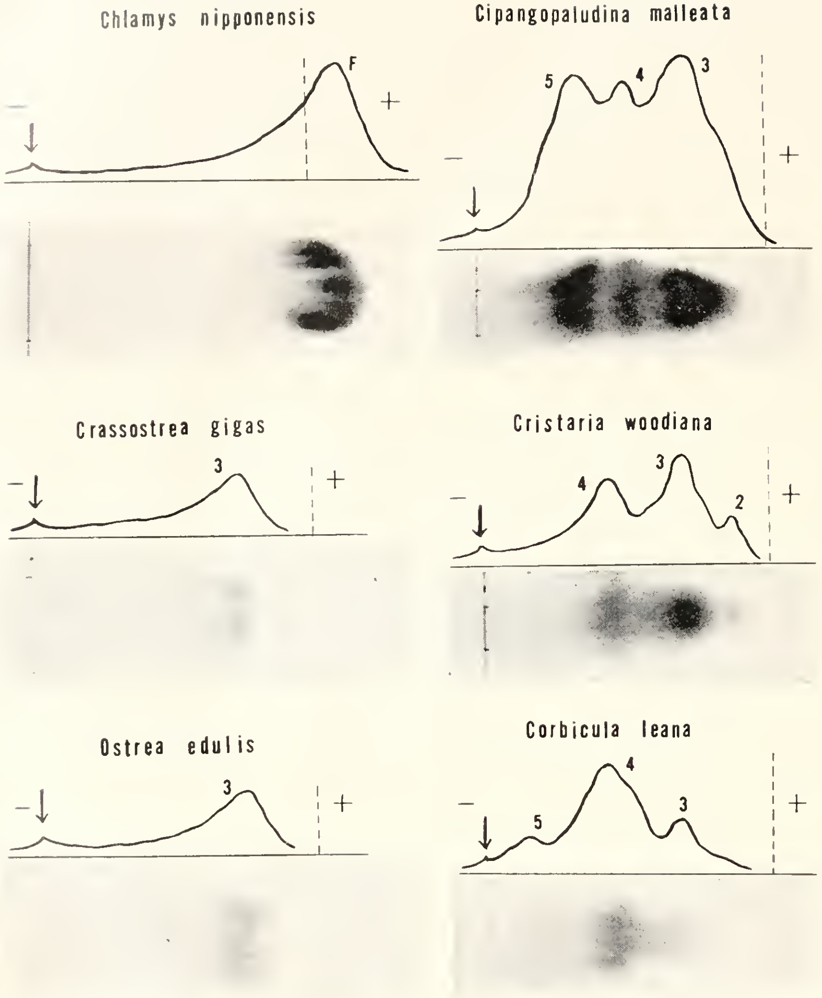


FIGURE 2. Paper electrophoretic patterns of the fluid proteins in six molluscan species. Dotted line indicates the relative migration distance of albumin of human serum.

cellulose acetate electrophoresis showed a relatively clear separation, although some fractions were found to show trailing and further, strictly speaking, migration distances of some fractions were not comparable with those of the paper electrophoresis. Nevertheless, from both the scannings and the diagrams shown in Figures 1 and 2, one is able to identify the individual protein fractions in each species. The relative migration distances of the protein fractions for each species are indicated in Table I. The variation in the migration rate of each fraction was considerable. However, except for the oysters, the species specificity of the electrophoretic patterns was always unmistakable. The migration rates of fractions of human serum are indicated in Table I for comparison.

A single protein fraction was found in the extrapallial fluid of *Aequipecten irradians concentricus, Chlamys nipponensis, Crassostrea virginica, C. gigas, Ostrea edulis,* and *O. lurida* (Figs. 1 and 2; diagram of the last species not presented). The occurrence of such a single fraction in these species was remarkably constant in every run, although it usually occupied a considerable area rather than appearing as a clear band. The fraction in the scallops, *Aequipecten irradians concentricus* and *Chlamys nipponensis,* corresponded to the so-called F-fraction, though the migration velocity of the former species was much faster than that of the latter (Table I). In all of the oysters, *Crassostrea virginica, C. gigas, Ostrea edulis* and *O. lurida,* the fraction was found at a position similar to alpha-2-globulin of human serum. Accordingly, it is probable that the two fractions of the two groups, scallops and oysters, differ chemically. This is further suggested by a characteristic difference in pattern shape on cellulose acetate (Fig. 1 B); that is, in *Aequipecten irradians concentricus,* the fraction was always found to show trail-

TABLE I

Relative migration distance of each protein fraction in extrapallial fluid and blood of fourteen molluscan species, calculated from the scannings of paper electrophoresis

Species	Fluids	No. of samples used	Protein fractions						
			F	1	2	3	4	5	6
Aequipecten irradians concentricus	Ex	14	144 ±9.4						
	Bl	9	123 ±5.4						
Chlamys nipponensis	Ex	8	107 ±7.1						
	Bl	10	111 ±5.1						
Crassostrea virginica	Ex	19				71 ±6.6			
	Bl	12				69 ±5.5			
Crassostrea gigas	Ex	9				68 ±3.9			
	Bl	2				66			
Ostrea edulis	Ex	7				67 ±3.1			
	Bl	2				70			
Ostrea lurida	Ex	4				70 ±2.2			
Busycon carica	Ex	17		94 ±4.8			44 ±6.3	16 ±3.0	
Elliptio complanatus	Ex	17			85 ±6.1	60 ±6.0	25 ±4.1		
	Bl	9			85 ±7.0	57 ±8.8	19 ±6.1		
Cristaria woodiana	Ex	19			86 ±5.5	66 ±5.8	36 ±3.2		
	Bl	5			81 ±3.0	64 ±1.8	33 ±6.5		
Corbicula leana	Ex	6				65 ±2.0	44 ±4.4	16 ±2.6	
Viviparus intertextus	Ex	8				64 ±4.6	48 ±5.2	17 ±1.5	
Cipangopaludina malleata	Ex	6				70 ±2.9	50 ±2.9	30 ±5.8	
Mercenaria mercenaria	Ex	20		98 ±4.5		41 ±7.1	19 ±4.2	11 ±3.5	
	Bl	9			85 ±5.2	39 ±4.6	23 ±4.5	9 ±2.2	
Modiolus demissus	Ex	15		95 ±6.5			24 ±7.3	10 ±3.5	−12 ±5.4
Human serum (control)		42		Alb 100	α_1 84 ±2.7	α_2 62 ±3.6	β 38 ±3.4	γ 11 ±2.0	

Fluids: Ex, Extrapallial fluid; Bl, Blood.
For each fraction, the value (±S.D.) for the migration distance is given relative to that of human serum albumin as 100%.

ing on cellulose acetate electrophoresis which was made under the same conditions as for *Crassostrea virginica.*

In each of the seven species, *Busycon carica, Elliptio complanatus, Viviparus intertextus, Cristaria woodiana, Corbicula leana,* and *Cipangopaludina malleata,* three fractions were found (Figs. 1 and 2). In all diagrams of *Busycon carica,* fraction 1 was found to be so faint as to be scarcely visible on paper after exposure to ammonia vapor, though it was clearly detectable on cellulose acetate strips. In almost all diagrams of *Elliptio complanatus* obtained by the cellulose acetate electrophoresis, the fastest fraction appeared to resolve itself into two fractions (Fig. 1 B). Although the occurrence of such an additional fraction was not distinctly recognizable in paper electrophoresis, four protein fractions may be present. In *Viviparus intertextus,* fraction 3 was not clear-cut on paper but was a distinct narrow band on cellulose acetate (Fig. 1 B). In all the Japanese three species, *Cristaria woodiana, Corbicula leana,* and *Cipangopaludina malleata,* three protein fractions were clearly identified, each species showing the respective specific pattern (Fig. 2). In the last species, the fastest fraction appears to resolve itself into two fractions when considered from its scanning pattern.

Among the 14 species studied, the electrophoresis of the extrapallial fluid in *Modiolus demissus* was most difficult. No satisfactory pattern was obtainable, possibly owing to its low concentration of total protein. And when the sample was concentrated more than 15 times, it was not easily absorbed on the filter paper, and especially on the cellulose acetate strip, because of its high viscosity. However, the scanning pattern of this species shown suggests four protein fractions, including one (fraction 6) which migrated towards the anode (Fig. 1 A). In most cases of paper electrophoresis in this species, fraction 1 was found to be so faint as to be scarcely visible after exposure to ammonia vapor, but it appeared as a relatively clear and slender band on cellulose acetate strips (Fig. 1 B). In *Mercenaria mercenaria* also, the four protein fractions are suggested but cannot be clearly resolved. Fraction 1, a minor component, scarcely detectable on paper, was always found to show trailing as a characteristic long tail-like appearance on cellulose acetate (Fig. 1 B).

The number of protein fractions in the blood was the same as in the extrapallial fluid in the eight species examined. However, as indicated in Table I, in *Aequipecten irradians concentricus* and *Mercenaria mercenaria* there were differences in migration velocity between blood and extrapallial fluid. The pericardial fluid of *Chlamys nipponensis, Crassostrea gigas,* and *Ostrea edulis* showed a single fraction is in both the extrapallial fluid and the blood in each species (diagrams not presented).

DISCUSSION

It is clear from the present experiments that the extrapallial fluid in the species with a calcite shell (*Aequipecten, Chlamys, Crassostrea,* and *Ostrea*) contains a single protein fraction, while species with an aragonite shell (*Busycon, Mercenaria, Elliptio, Viviparus, Cristaria, Corbicula,* and *Cipangopaludina*) or a shell of both aragonite and calcite (*Modiolus*) have three or more protein fractions. The species studied are few in number; however, these results suggest the possibility that extrapallial fluid which possesses a complex system of protein components may favor aragonite formation, while that having a single protein component may crystallize as calcite during shell deposition.

Yago *et al.* (1959) have reported that both the blood and the pericardial fluid in *Crassostrea gigas* revealed a single protein fraction, in agreement with the present experiments. Woods *et al.* (1958) have reported that the serum of *Crassostrea virginica* showed two slow components, using starch gel electrophoresis. However, the writer's preliminary experiments made on the same species suggest that some slow components are often found to occur in the following cases: in extrapallial fluid collected from a specimen in which the mantle was intentionally injured by the tip of the needle, and in blood collected from a relatively small heart by strong suction with the syringe. Further, paper electrophoresis of minced tissue fluid in this species also showed the occurrence of such slow components.

The technique of paper partition chromatography has been applied to the taxonomic study of molluscs. Wright (1959) and Collyer (1961) have reported the biochemical specificity in mucus and tissue fluid. The ninhydrin-positive substances studied by these investigators, and protein components in the extrapallial fluid, showed a remarkable degree of specificity except in the species of the oysters studied by electrophoresis. The specific fenestration of the organic matrix in the shell (Grégoire *et al.*, 1955) might be a pattern formed in relation to such a biochemical specificity of the extrapallial fluid in molluscs. Accordingly, both *in vitro* and *in vivo* studies which may show a correlation between the organic matrix, as synthesized from either a simple or a complex system of protein components and the crystal type of calcium carbonate, may offer some evidence on the mechanism of shell formation. It is interesting to note here that such a preliminary *in vitro* experiment has been made by Kitano and Hood (unpublished data).

Summary

1. Distribution of the protein fractions of the extrapallial fluid of fourteen species of molluscs was determined by means of paper and cellulose acetate electrophoresis.

2. The extrapallial fluid of species in which the calcium carbonate of the shell occurred as calcite contained a single protein fraction. In those species in which the shell calcium carbonate occurred as aragonite or as both aragonite and calcite, three or more protein fractions were present in the extrapallial fluid.

LITERATURE CITED

Bøggild, O. B., 1930. The shell structure of the mollusks. *Danske Vidensk. Selsk. Skr.*, **9**, II-2: 232-326.

Bronn, H. G., 1935. Klassen und Ordnungen des Tierreichs, **3**(3): 306–509.

Collyer, D. M., 1961. Differences revealed by paper partition chromatography between the gastropod *Nassarius reticulus* (L.) and specimens believed to be *N. nitid* (Jeffreys). *J. Mar. Biol. Assoc.*, **41**: 683–693.

de Waele, A., 1930. Le sang d'*Anodonta cygnea* et la formation de la coquille. *Mém. Acad. Roy. Belg., Cl. Sci.*, **10**: 1–52.

Grégoire, C., 1957. Topography of the organic components in mother-of-pearl. *J. Biophys. Biochem. Cytol.*, **3**: 797–808.

Grégoire, C., G. Duchateau and M. Florkin, 1955. La trame protidique des nacres et des perles. *Ann. Inst. Océanog.*, **31**: 1–36.

HARE, P. E., 1963. Amino acids in the proteins from aragonite and calcite in the shells of *Mytilus californianus*. *Science*, **139**: 216–217.

HOLT, V. C., K. GAEDE AND K. D. VOIGT, 1962. Papierelektrophorese von Eiweisskörpern bei erhöhter Spannung. *Biochem. Zeitschr.*, **323**: 345–354.

LOWENSTAM, H. A., 1954. Factors affecting the aragonite:calcite ratios in carbonate-secreting marine organisms. *J. Geol.*, **62**: 284–332.

ODUM, H. T., 1957. Biochemical deposition of strontium. *Inst. Mar. Sci.*, **4**: 38–114.

RAYMOND, S., 1955. Paper Electrophoresis. E-C Apparatus Co., Swarthmore, Penna.

ROBERTSON, J. D., 1941. The function and metabolism of calcium in the Invertebrata. *Biol. Rev.*, **16**: 106–129.

ROCHE, J., G. RANSON AND M. EYSSERIC-LAFON, 1951. Sur la composition des scleroproteines des coquilles des mollusques (conchiolines). *C. R. Soc. Biol.*, **145**: 1474.

SMITH, I., 1960. Chromatographic and Electrophoretic Techniques. II. Zone Electrophoresis. William Heinemann-Medical Books, Ltd., London, and Interscience Publishers, Inc., New York.

STOLKOWSKI, J., 1951. Essai sur le détermisme des formes minéralogiques du calcaire chez les étres vivants (calcaires coquilliers). *Ann. Inst. Océanog.*, **26**: 1–113.

TANAKA, S., H. HATANO AND O. ITASAKA, 1960. Biochemical studies on pearl. IX. Amino acid composition of conchiolin in pearl and shell. *Bull. Chem. Soc. Japan*, **33**: 543–545.

TUREKIAN, K. K., AND R. L. ARMSTRONG, 1960. Magnesium, strontium, and barium concentrations and calcite-aragonite ratios of some recent molluscan shells. *J. Mar. Res.*, **18**: 133–151.

WADA, K., 1961. Crystal growth of molluscan shells. *Bull. Natl. Pearl Res. Lab.*, **7**: 703–828.

WATABE, N., AND K. M. WILBUR, 1960. Influence of the organic matrix on crystal type in molluscs. *Nature*, **188**: 334.

WOODS, K. R., E. C. PAULSEN, R. C. ENGLE, JR. AND J. H. PERT, 1958. Starch gel electrophoresis of some invertebrate sera. *Science*, **127**: 519–520.

WRIGHT, C. A., 1959. The application of paper chromatography to a taxonomic study in the molluscan genus *Lymnea*. *J. Linn. Soc. (Zool.)*, **44**: 222–237.

YAGO, N., K. NAGASE, M. YAMAGUCHI AND S. TAKATSUKI, 1959. Studies on the chemical constitutions of the body fluids in the oyster. I. On the proteins of the blood and pericardial fluid in *Gryphea gigas*. *Zool. Mag. Japan*, **68**: 386–389.

YASUDA, M., M. TSUCHIYA AND T. UEDA, 1957. Amino acid composition in pearl and shell of pearl-oyster, *Pinctada martensii*. *Bull. Fac. Fish., Nagasaki Univ.*, **5**: 121–127.

VARIATIONS IN TIME AND INTENSITY OF SETTING OF THE STARFISH, ASTERIAS FORBESI, IN LONG ISLAND SOUND DURING A TWENTY-FIVE-YEAR PERIOD

VICTOR L. LOOSANOFF

U. S. Bureau of Commercial Fisheries, Biological Laboratory, Milford, Connecticut[1]

The common starfish, *Asterias forbesi* (Desor), is found in greatest concentrations in the waters of southern New England, principally in Long Island Sound. The latter is a semi-enclosed basin approximately 100 nautical miles long and occupying an area of about 925 square miles. The Sound's greatest width of about 17 miles is along the line drawn perpendicular to the Long Island shore from Branford, Connecticut. The width decreases gradually from this line in both directions, being confined eventually to only about one mile at its west entrance and to approximately eight miles at the eastern end.

The average depth of the Sound is approximately 60 feet, and the greatest depth of about 306 feet, recorded during our surveys, was near the Sound's extreme eastern end, a few miles southwest of Fishers Island. The eastern part, in general, is somewhat deeper than the western, while the middle and the deepest part of the Sound is relatively level and averages about 90 feet in depth (Loosanoff and Engle, 1940). Information on various aspects of oceanography of Long Island Sound is given by Riley *et al.* (1956).

Surveys of the occurrence and distribution of starfish in Long Island Sound made in different years and during different seasons have shown that these echinoderms were present in all depths from a few feet above the low water mark to 250 feet, although most of them were found near the shores in comparatively shallow water, usually less than 50 feet (Galtsoff and Loosanoff, 1939). These surveys also demonstrated that in Long Island Sound there were no seasonal migrations of starfish from shallow to deep water or *vice versa.* Their distribution was primarily influenced by the location and numbers of bottom organisms on which they feed.

Because of their common occurrence in New York and New England waters, where many centers of research in marine biology of our country originated, the starfish were subjects of studies of many biologists, including Agassiz (1877), Clark (1904), Mead (1901), and Coe (1912). However, the literature dealing with the natural history of *A. forbesi* does not offer comprehensive accounts of time and intensity of setting of this invertebrate. With the exception of a few sentences in Mead's (1901) article on starfish of Narragansett Bay, Massachusetts, and several remarks in papers of Loosanoff (1936) and Galtsoff and Loosanoff (1939) on setting of starfish in Long Island Sound, no other reports are available. Yet, considering the common occurrence of *A. forbesi* and, especially, its importance as an enemy of commercial mollusks, principally oysters, which begin to set approximately two weeks later than the starfish (Loosanoff and Engle, 1940), such information is obviously not only of theoretical but also of practical interest and importance.

---

[1] Present address: U. S. Bureau of Commercial Fisheries, Tiburon, California.

MATERIALS AND METHODS

To acquire more information on setting of starfish a study of this phase of the animal's life history was undertaken in 1937 and continued without interruption for a period of 25 years, until 1961. The observations were largely confined to the northern part of Long Island Sound, along the Connecticut shore. In some years, especially during the early part of our observations, the chain of stations established for collection of samples extended from the Thimble Islands to Westport. These stations, the number of which varied from 3 to 23 in different years, were located at depths ranging from intertidal zones to about 100 feet. Since 1944, however, with the exception of the year of 1957, observations were made at the same ten stations located at the depths of about 10, 20, and 30 feet at mean low water mark in the area extending from Bridgeport to New Haven (Fig. 1).

The results presented in this paper are based upon the data obtained from the stations located at the depths of 30 feet or less. Because of this decision, the question naturally arose whether these data would also be representative of the time and intensity of setting of starfish at greater depths of the Sound. The answer was provided by an analysis of our material collected during 1938, when a series of 10 stations was established near Stratford Point, commencing at mean

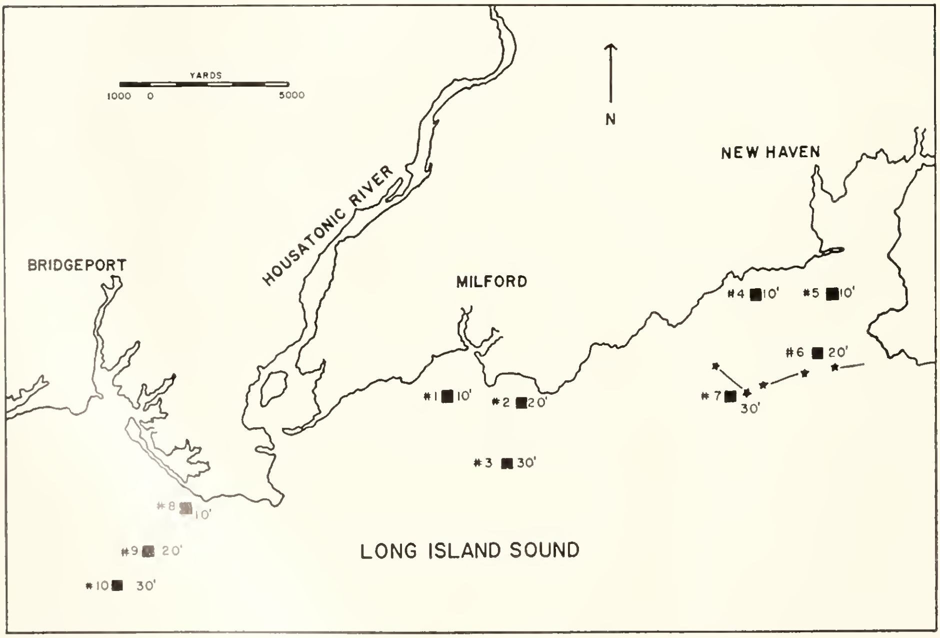


FIGURE 1. Locations and depth in feet of 10 basic stations established in Long Island Sound for observations on time and intensity of starfish setting, 1944–1961.

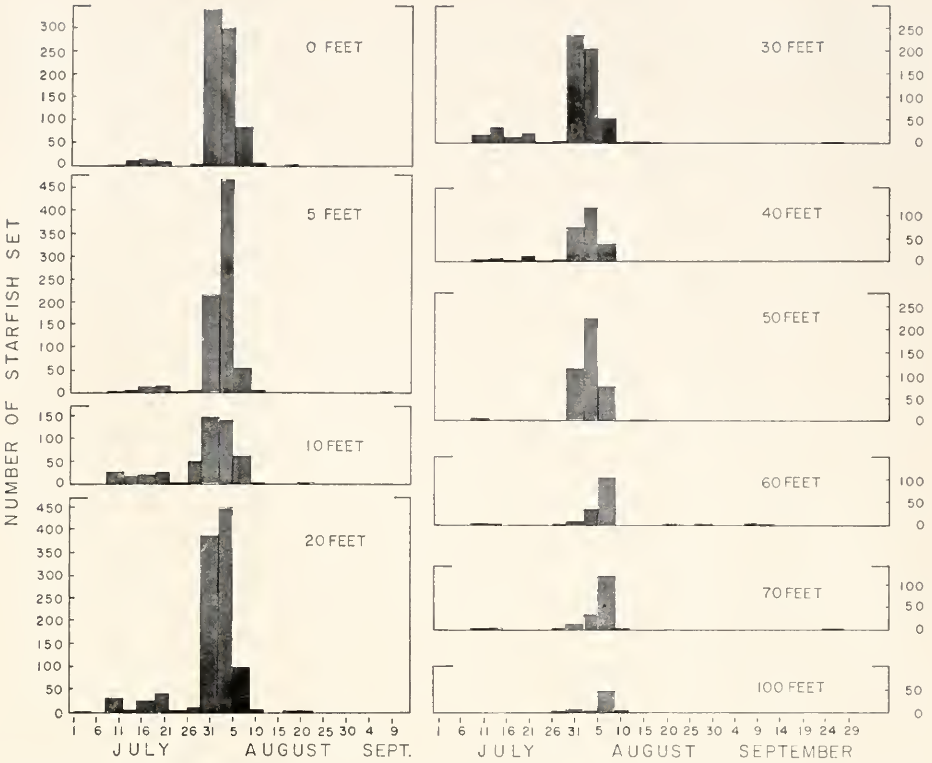


FIGURE 2. Numbers of starfish set on one hundred oyster shell-surfaces, recorded at the stations located at 10 different depths off Stratford Point. Long Island Sound, summer, 1938.

low water mark, where the Housatonic River enters the Sound, and extending almost to the middle of Long Island Sound to the depth of 100 feet. The collectors were placed at all the stations on July 17, but no set occurred during that month (Fig. 2). The first set was found between July 1 and 5, but it was extremely light, occurring on only one collector at the 20-foot station. By July 12, however, starfish set was found at all stations except the one at 100 feet, where the first set was recorded somewhat later.

The setting at the deepest station was lighter than at any of the more shallow ones, and the duration of the setting was shorter. Nevertheless, the beginning of setting at such depths as 50, 60, and 70 feet coincided with that at the shallow stations. Furthermore, the period of heaviest setting at all stations occurred almost simultaneously (Fig. 2).

The setting ended virtually at the same time at the shallow and at the deeper stations; the last few starfish were found on September 28 at 30-foot station and at the station located at 70 feet. In 1937, nevertheless, when the series of stations in the Stratford Point area extended to a depth of only 70 feet, several single starfish were noticed on the collectors at deeper stations after the setting at more shallow stations had already ended. However, these individuals were so few that their presence did not modify the general pattern of setting. Therefore, it may be concluded that, even though our observations on the time and intensity of setting were conducted only at stations located at depths of 30 feet or less, they were, nevertheless, quite representative for the general starfish population of Long Island Sound especially if it is remembered that most of the starfish are found near the shore in comparatively shallow water.

Our studies were designed to determine, for each year, the date of the beginning of setting, end of setting, duration of the setting period, and variations in the intensity of setting throughout the summer. Effects of several environmental factors, including temperature and salinity of the water, on the above-mentioned chronological events, and in relation to the intensity of setting within the same year and during different years, were also evaluated to provide, if possible, a reliable basis for predicting the time and intensity of setting. However, this part of our studies, representing extensive statistical analyses of the numerous variables and factors involved in propagation of starfish, will be described in another article.

The time and intensity of setting in different areas of Long Island Sound were ascertained by examining and counting starfish set on the collectors placed on the bottom. These collectors were the same type as those used for observations on setting of oysters. Since they have already been described in detail (Loosanoff and Engle, 1940), no extensive description is offered here. It is sufficient to mention that they were wire-mesh bags filled with clean oyster shells. The bags were of the same dimensions and contained approximately the same number of shells selected for their uniformity.

Twice a week, collectors at all stations were changed and brought to the laboratory to be examined. Biologists counting the recently set starfish were trained to distinguish the individuals of different ages from the time they metamorphosed to the time they were 4 or 5 days old. The counts were made on the inside surfaces of 20 shells taken from each collector, and the results were later averaged and daily intensity of setting estimated per 100 shell-surfaces. This is the criterion on which all the data offered in this article are based.

When it was decided to try this method, we suspected that a significant error might be introduced in obtaining true figures because many recently attached starfish would be washed off the shells as the collectors were brought up through many feet of water. Fortunately, the following test showed that young starfish attach themselves very securely to the shells and, therefore, are not easily washed off the collectors. A number of shells with starfish set attached were taken from collecting bags and circles were drawn in pencil around each starfish. Immediately afterward, the shells were returned to the bags and the latter were lowered to the bottom. The bags were then raised, after which the shells were re-examined and the number of empty circles, indicating washed-off starfish, counted. This test demonstrated that only a few starfish were lost.

To secure more reliable data for our general studies, two bag-collectors were used at each station. Later on, when extensive material was already available, we decided to verify once more, by employing the following analysis, the reliability of our method of using two bags at each station. Two years of fairly heavy starfish setting, 1941 and 1952, were selected and correlations between bag A and bag B were computed for each collection date on which there were at least 10 starfish in one or more bags. For 1941 these correlations ranged from 0.58 to 0.97; for the year 1952, from 0.59 to 0.98. The over-all average correlation was 0.88 and the estimated reliability, based upon data from both bags, was 0.94. This is a highly satisfactory reliability figure indicating, therefore, that even during times when at some stations one of the two bags was lost, our results on the intensity of setting for that period based on examination of only one bag were still reliable.

In those instances when at some stations both bags were lost due to severe storms or because the floats indicating the position of the bags were cut off by the propeller of a passing boat, the figures of the intensity of setting for the elapsed period for those stations were arrived at by averaging the number of set at the two nearest stations.

Results

According to Mead (1901) the height of the spawning season of *A. forbesi* in Narragansett Bay took place between June 4 and June 16. By the end of June many starfish in that area had extruded most of their spawn but, nevertheless, individuals with apparently undischarged gonads were occasionally found even in July. Agassiz (1877) stated that *A. forbesi* of northern waters began to spawn only during the last part of July. Coe (1912) found that starfish in Long Island Sound matured their sex products in May and early June and spawned during late June. Coe, however, emphasized considerable individual variations to be found among the starfish. A closely related form, *Asterias vulgaris*, common on the Atlantic coast of Canada, spawned, according to Smith (1940), in early June. Hancock (1958), working on Essex oyster beds in England in 1954, found that *A. vulgaris* began to spawn on May 29 when the water temperature had reached 15.0° C. Another closely related species, *Asterias rubens*, began to spawn as early as April (Vevers, 1949). A more extensive review of the spawning of asteroids of the north temperate zone is offered by Hyman (1955).

Our observations on *A. forbesi* in Long Island Sound, covering the period of more than two decades, showed that, in general, they commence to spawn within a few days after the water temperature reaches approximately 15.0° C., which usually occurs around the middle of June. As observed by Mead in Narragansett Bay, our starfish usually do not discharge their entire supply of sex products within a few days but, on the contrary, some individuals spawn intermittently until the end of August and, in certain cases, spawning extends into early September.

After completion of spawning the gonads of starfish undergo the process of resorption, usually complete in October, after which active development of new sex cells begins. Gametogenesis is quite rapid during November and December and, as a result, the gonads increase considerably in size during that period. Between January and March many starfish may already be found with gonads of nearly normal size, although they are probably physiologically immature because, in most instances, the eggs obtained from the ovaries of the starfish at that time of the year could not be fertilized. However, by following our method of conditioning bivalves for spawning outside their natural period of propagation (Loosanoff and Davis, 1950), development of gonads of starfish in late winter and early spring can sometimes be accelerated.

The length of the larval period of *A. forbesi* under natural conditions has never been precisely ascertained. Our field observations and laboratory experiments indicate, nevertheless, that in early summer its length may be approximately three weeks. For example, observations made in 1937 showed that the first spawning of starfish in Long Island Sound occurred on June 16 and the first set was recorded about July 8, or approximately 22 days later. In 1938 the first spawning was observed on June 14, whereas the first setting, although very light, was noted about July 5, again approximately 21 or 22 days later. The field observations on the duration of the larval period were in agreement with the results of our laboratory experiments, where the first metamorphosing larvae in our cultures were noticed 20 or 21 days after fertilization.

It is realized that even though the period elapsing between the time of fertilization of the egg and metamorphosis of the larva was established, during our field and laboratory observations, as about 21 days, this period, as in the case of larvae of other invertebrates, for example, oysters, *Crassostrea virginica*, or clams, *Mercenaria mercenaria*, may vary considerably according to changes in the environment, such as changes in temperature or in quality or quantity of food (Loosanoff and Davis, 1963). For example, whereas larvae of *M. mercenaria* grown at a temperature of about 30° C. metamorphosed in some instances within 7 days after fertilization, larvae originating from the same parents but kept at a temperature of 18° C. required as long as 16 or even 24 days before they began to set. Thus, it is logical to assume that, while starfish larvae originating from early spawning in June, when the water temperature in Long Island Sound is normally only slightly above 15° C., require approximately three weeks to reach metamorphosis, larvae originating from eggs discharged in the middle of summer, when the temperature of 20° C. or higher prevails, may develop in half the time indicated above, especially if good food organisms needed for larval growth and development are present in optimum numbers.

Under adverse environmental conditions, such as periods of low temperature following discharge of eggs or relative sparseness of good food organisms, the larval stage may be of much longer duration. Periods of heavy rains causing reduction of salinity of the water to approximately 16–18 ppt., the lowest level at which normal development of starfish may proceed (Loosanoff, 1945), may also be responsible for prolongation of the larval period or even for complete mortality and, therefore, disappearance of starfish eggs and larvae.

Mead (1901) stated that in Narragansett Bay starfish first began to set June 28, or within a day or two of that date. He, however, did not give any figures showing intensity of setting. Mead also found that in that locality starfish set principally during the last few days of June and the first week of July, but the setting sometimes continues as late as July 16. As will be shown later, the average length of the setting period in Long Island Sound is considerably longer than that reported by Mead.

Our observations indicated that, with the exception of the year 1945, when setting began and ended within the same day, September 11, the date of beginning of setting varied in different years from the last week of June to the last week of July (Fig. 3). However, since these observations were made primarily at stations located in open Long Island Sound at depths ranging from 10 to 30 feet, it is pos-

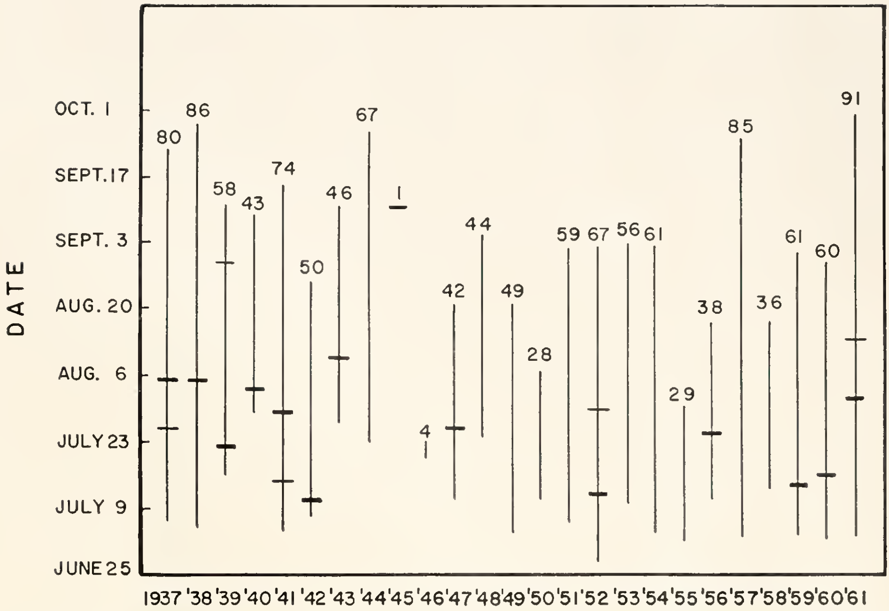


FIGURE 3. Length of setting period of starfish in different years. Cross-bars indicate the time when peaks of first and second waves of setting occurred, broader bars indicating the peaks of the heavier waves. Long Island Sound, 1937–1961. Figures at top of each bar indicate number of days in each setting period.

sible that, in some inshore, shallow, well-protected areas, where the temperature of the water is somewhat higher, scattered light setting may have preceded that of Long Island Sound proper by a day or two, a circumstance not substantially changing the basic annual picture of setting.

During the 25-year period, in almost half the years the beginning of setting occurred before July 8, while in only three years did it occur as late as the last week of July. During the remaining years, except 1945 when setting was abnormally delayed and confined to September 11 only, the beginning of setting took place between the end of the first and the beginning of the last week of July. In the years when intensity of setting was medium or heavy, the beginning of setting usually occurred within the same 24 hours at all three depths.

Setting in different years ended between July 23 and October 1 (Fig. 3). It extended into the second part of September in only five years. During four of these years setting began early and the setting period was, therefore, of long duration. The exception was 1944 when the beginning of setting was delayed until July 23.

One of the most significant observations made during these studies concerns the length of the setting period in different years. In 1945 setting began and ended within the same day. In other years its length varied from about 4 days in 1946, a year of very light setting, to 91 days in 1961 (Fig. 4). In four years setting continued for 80 days or longer. The average annual length of the setting period was about 52 days. However, considering extensive deviations from this figure in both directions, it is given here merely as an approximation.

The earliest date for the beginning of setting was June 28, in 1952, and the latest, July 29, in 1940. Thus, even not considering the abnormal year 1945, the date for the beginning of setting of starfish in Long Island Sound differed by 31 calendar days, a rather extensive period. The end of setting in different years occurred between July 23 and October 1, covering an even longer period of approximately 70 days, thus indicating the great variations in the chronology of annual biological events involved in the propagation of starfish.

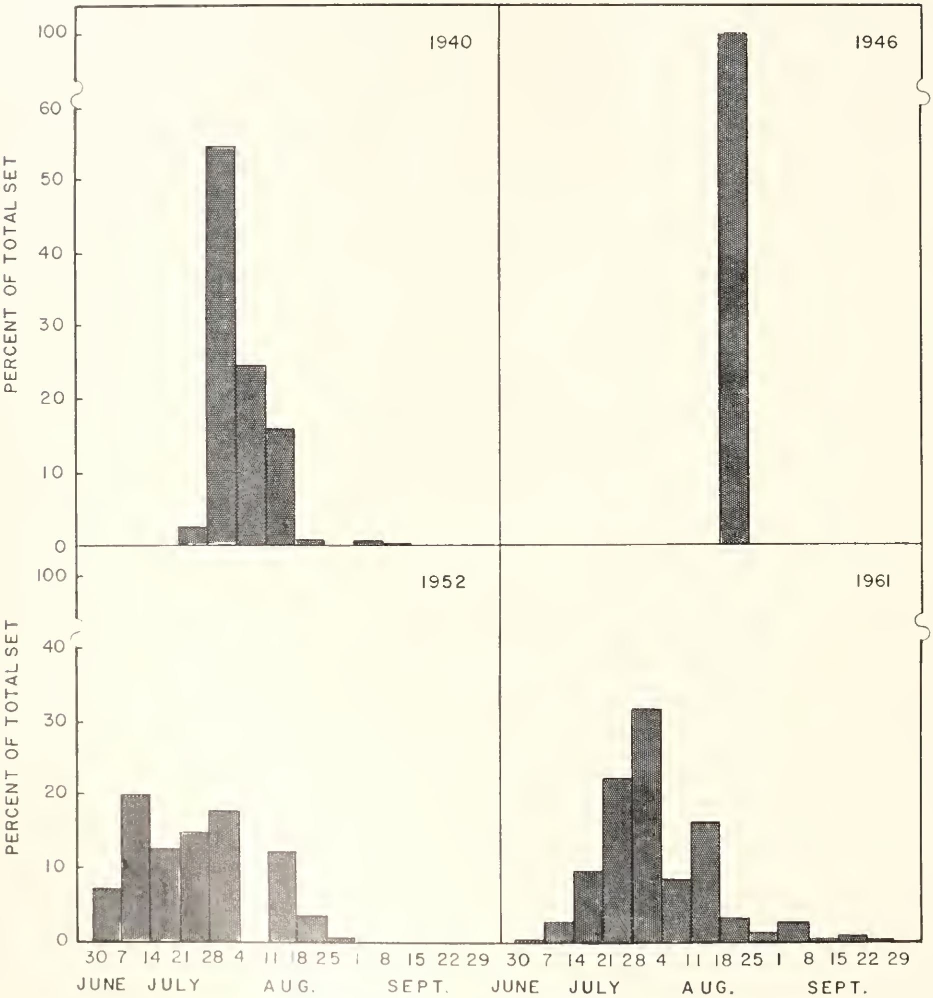


FIGURE 4. Variations in weekly intensity of starfish setting in different summers. Sometimes, as in 1946, the entire set (100%) may occur within a single week, while in other years, as 1961, when setting continues for a long time, its intensity may vary greatly from week to week.

For organisms like the American oyster, *C. virginica*, setting of which is usually relatively heavy, the date when the maximum setting of the season occurs may be ascertained with comparative ease (Loosanoff and Nomejko, 1951). In the case of starfish, however, such maxima are not always clearly defined because the intensity of setting is too light or, in some instances, too uniform to show distinct waves of setting culminated by definite peaks. In the years of heavy setting, however, not one, but two distinct waves of setting may, nevertheless, be recorded. Such quite clearly defined double waves occurred in 1937, 1939, 1941, 1952, and 1961 (Fig. 3). In three of these years the first wave was the heavier of the two, but in the remaining two, 1937 and 1941, setting began with a light wave while the second and heavier wave occurred later in the season.

Searching for signs of periodicity, or orderly patterns in the chronology of events of starfish setting, we found that the interval extending from the beginning of setting to the peak of the first wave, recorded during the 13 years when such peaks could be clearly established, varied in different years from 3 to 31 days, but showed no definite pattern. During the five years when the maxima of the first and second waves were determined, the intervals between the two peaks were

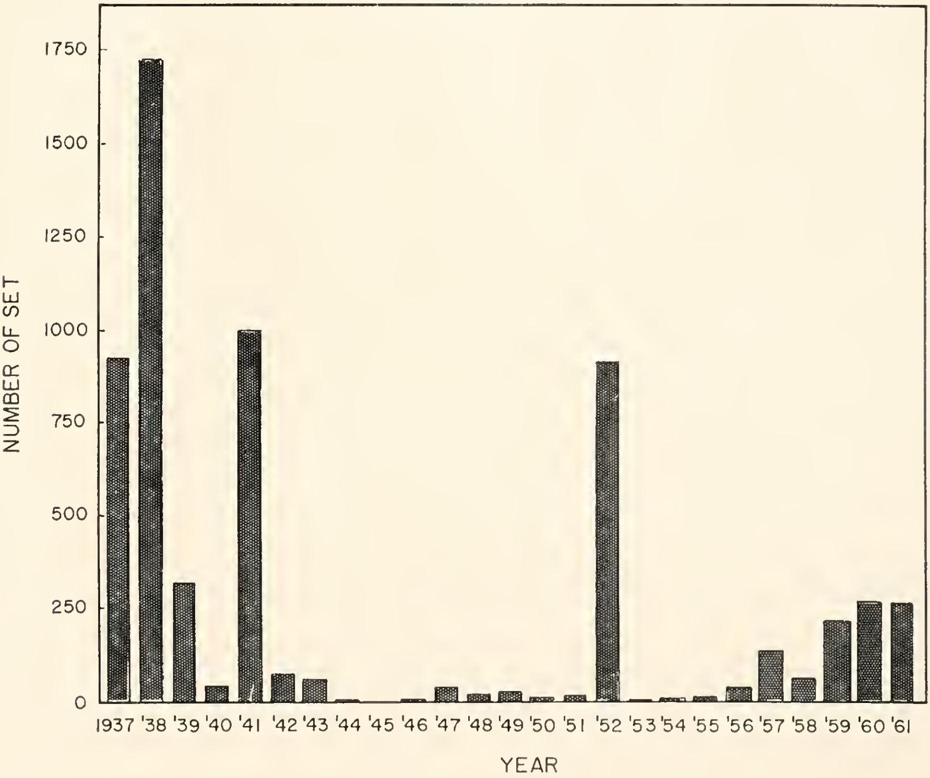


FIGURE 5. Average number of starfish set per station recorded on one hundred oyster shell-surfaces during different years. Long Island Sound, 1937–1961.

10, 39, 14, 32, and 49 days, again suggesting no periodicity. Finally, in the same five years, the beginning of the second wave occurred 30, 45, 25, 20, and 12 days after the beginning of setting, again failing to indicate any pattern.

As can be expected over such a lengthy period of observation, considerable variations in intensity of setting were recorded from year to year (Fig. 5). The rank orders of each of 25 years, based on averaging the intensity of setting each year for all the stations, regardless of their depth, and for the groups of stations located at the three different depths are given in Table I. The year 1938 ranked highest, while 1945 was lowest. Ranks of other years, as given in Table I and as shown, indirectly, in Figure 5, indicate that there was no definite succession of good and poor years. For example, the good years, such as 1941, may be preceded by a year of relatively poor setting while, on the other hand, the years of poor setting, such as 1942 and 1953, may be preceded by very good years.

TABLE I

*Yearly rank-order of intensity of starfish setting at stations of 10-, 20-, and 30-foot depths, and ranking average of all stations during each year of the 25-year period. Long Island Sound, 1937–1961**

YEAR	Rank-order of stations				YEAR	Rank-order of stations			
	10 ft.	20 ft.	30 ft.	All stations		10 ft.	20 ft.	30 ft.	All stations
1937	2	2	3	3	1950	21	16	20	20
1938	3	1	1	1	1951	22	22	17	19
1939	5	7	6	5	1952	4	3	2	4
1940	14	14	11	14	1953	25	24	24	24
1941	1	4	4	2	1954	20	21	23	22
1942	9	11	14	10	1955	19	19	19	18
1943	16	10	10	11	1956	12	18	12	13
1944	18	20	22	21	1957**	10	9	8	9
1945	24	25	25	25	1958	11	12	13	12
1946	23	23	21	23	1959	6	6	9	8
1947	15	13	15	15	1960	8	8	5	6
1948	13	17	18	17	1961	7	5	7	7
1949	17	15	16	16					

* In case of a tie, the earlier of the two years was given higher rank.
** Only stations 1, 2, and 3, all in the Milford area, were established.

Observations made during the period extending from 1944 to 1961, when the same 10 stations were used (Table II), as well as during the entire period of 25 years' study (Fig. 6), showed that, in general, during the years of heavy setting good sets were recorded at all depths. However, although the trend was not too well-marked, there was a tendency for the depths to be ranked in the following order: 30 feet—heaviest, 20 feet—medium, and 10 feet—lightest. The Bridgeport area was first, producing the heaviest sets for most of the years, while Milford and New Haven were quite consistently second and third, respectively (Table II).

Although, as mentioned earlier in this article, results of the statistical studies carried on to ascertain the presence or absence of simple or compound correlations existing between numerous biological and ecological variables involved in propagation of starfish will be discussed in another paper, it may be appropriate here to mention briefly some of our findings that are of general interest. For example, it was determined that sets beginning early in the season may be either heavy or light, but those beginning late are usually light. Thus, all the sets that began on July 20 or later, taking place in the years 1940, 1943, 1944, 1946, and 1948, were light.

While light sets may end early in the season, heavy sets normally end fairly late. Taking the years of heavy and medium-heavy setting, which includes the years 1937, 1938, 1939, 1941, 1952, 1957, 1959, 1960, and 1961, ranking in intensity of setting from 1 to 9 during the 25-year period (Table I), we find that,

Table II

Number of starfish set on 100 shell-surface at each of 10 stations as recorded in different years; rank-order of each station and each of the three areas for this period. Long Island Sound, 1944–1961

Areas	Milford			New Haven				Bridgeport		
Stations	1	2	3	4	5	6	7	8	9	10
Years	Numbers of starfish set									
1944	9	6	6	0	3	3	0	14	20	20
1945	0	0	0	0	0	0	0	0	0	3
1946	3	0	15	0	0	0	0	3	16	16
1947	33	60	76	0	3	6	33	18	128	67
1948	51	6	35	0	3	3	11	12	44	39
1949	15	33	79	0	0	0	16	16	62	79
1950	3	11	6	3	3	8	6	3	42	29
1951	0	5	33	0	0	0	28	12	15	29
1952	124	635	1605	19	74	357	721	1621	1597	2453
1953	0	0	3	0	0	0	0	0	3	9
1954	3	8	6	0	0	3	3	11	15	9
1955	3	12	23	3	0	6	14	21	21	34
1956	18	26	101	3	6	6	31	78	18	147
1957*	62	113	238	—	—	—	—	—	—	—
1958	85	50	65	0	0	0	0	144	191	156
1959	95	184	275	51	92	124	105	237	730	302
1960	64	200	536	88	82	164	798	102	277	379
1961	58	272	315	6	33	125	240	281	861	483
Station total	564	1508	3179	173	299	805	2006	2573	4040	4254
Station rank	8	6	3	10	9	7	5	4	2	1
Area average set per station	1750			319				3622		
Area rank	2			3				1		

* Since only stations #1, #2, and #3, all in the Milford area, were maintained in 1957, this year is not included in ranking estimates.

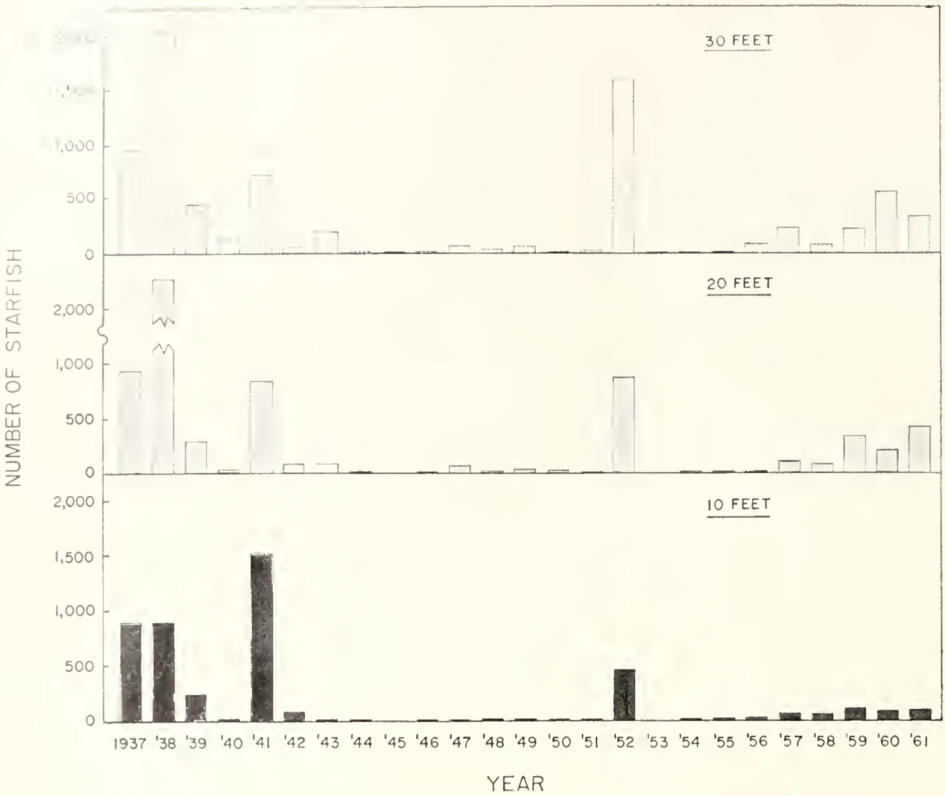


FIGURE 6. Average number of starfish set per station recorded in different years on 100 oyster shell-surfaces at stations located at 10-, 20-, and 30-foot depths. Long Island Sound. 1937–1961.

with the exception of 1960, setting during all these years extended into September. In four of these years setting continued almost until the end of September and, on one occasion, actually occurred on October 1.

Taking the ten poorest years, ranking from 16th to 25th, only in one year, 1945, did setting extend beyond the first week in September. However, in that unusual case setting began and ended on September 11. In some poor years, as in 1946, setting ended on July 23, while during another poor year, 1950, it ended on August 7. Thus, in general, during the years of heavy setting, starfish continued to set later in the season than during the years of light setting.

Heavy sets tend to be of long duration, while poor sets tend to be short. During the nine years of heavy sets the setting period ranged from 58 to 91 days, averaging about 74 days. The length of the period during the ten poorest years, however, ranged from 1 to 67 days and averaged only about 40 days.

A setting which reached a maximum, or peak, early in the season may end early or fairly late, while a setting that reached a maximum late tends to end late in the season. This tendency, however, was not too well-defined because in some years, for example, 1944 (Fig. 3), setting continued as late as September 27, even though no definite peaks or waves were manifested.

During the period of 17 years, from 1940 to 1956, Milford Laboratory conducted regular, semi-annual surveys on the distribution and occurrence of adult starfish over the oyster-producing grounds located along the Connecticut shore between Branford and Black Rock, west of Bridgeport Harbor. Uniform methods were used throughout the period, and the dredging was done along the same lines. Results of these surveys were reported in bulletins issued by our laboratory for the information of members of the Connecticut and New York oyster industries. The reports were accompanied by maps showing the lines of the surveys and the numbers of starfish found along these lines. In preparation of this article it was decided to use these data to compare the intensity of starfish setting in different years with the numbers of adult starfish recorded during the spring surveys, the time corresponding to the pre-spawning period. The relative abun-

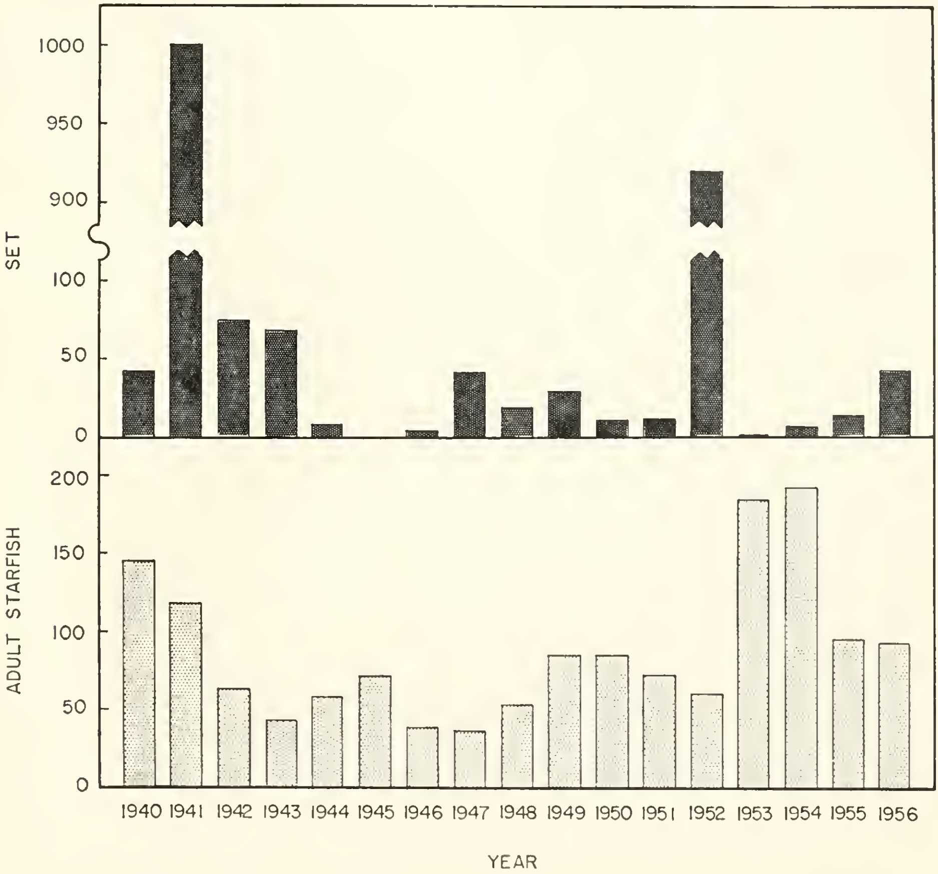


Figure 7. Comparison between numbers of adult starfish and starfish set in different years. Long Island Sound, 1940–1956.

dance of adult starfish was based on the average number of these echinoderms per 10,000 feet of dredging. This approach was shown statistically to be the most reliable measure, as compared to others, such as average number of starfish per 10-minute drag, etc.

As has already been shown, there was considerable variability in the numbers of starfish set in different years, with only two good years recorded during the period extending from 1940 until 1956 (Fig. 7). The numbers of adult starfish, recorded in different years during the same period, showed much less variability. In general, the comparison indicated no appreciable relationship between numbers of adult starfish existing during pre-spawning periods and intensity of starfish setting later in the season. For example, while a heavy set of starfish in 1941 was preceded by a spring showing a high adult population, the other season of heavy setting, 1952, was characterized by relatively low numbers of adults. Moreover, even though in 1953 and 1954 the numbers of adult starfish recorded were the highest during the period of observations, the starfish sets during these two years were among the lowest on record (Fig. 7).

It is also of considerable biological interest that, regardless of a very heavy starfish set in 1941, the adult population of starfish in 1942 showed almost a 50% decrease, instead of a normally anticipated increase. We have already reported that, similar to the observations described in this article, we found no relationship between the number of adult oysters and intensity of oyster setting in Long Island Sound (Loosanoff *et al.*, 1955).

Burkenroad (1946), basing his conclusions chiefly on newspaper notes and conversations with oyster growers, concluded that the starfish has been particularly destructive to the oysters of Long Island Sound at intervals of about 14 years. According to him such peaks of population have occurred in 1900, 1914, 1928, and 1942. Adding 14 years to the last figure the year 1956 should then be another year of population peak. Our data show, nevertheless, that neither 1942 nor 1956 was a year of good starfish setting (Figs. 5 and 7). They ranked only number 10 and number 13, respectively, thus being about average years (Table I). Therefore, our observations on intensity of setting do not support Burkenroad's contention. It is realized, nevertheless, that as we have just mentioned, the increases in numbers of adult starfish in Long Island Sound are not proportional to the numbers of set because, as is often the case in such forms as starfish and oysters, a heavy set may perish within a few days or weeks after its occurrence, thus contributing nothing to the increase of the adult population; while, in other years, a relatively light set that survives well may considerably increase the numbers of adults.

We may add to the above discussion that our field studies show that neither of the years 1942 or 1956 was characterized by large populations of adult starfish (Fig. 7). Furthermore, the year of 1949, which according to Burkenroad's estimates should have been the year when adult starfish were least numerous, was in reality a year of larger population than the year of 1942 and of only somewhat smaller population than the year of 1956. Coe (1956), discussing fluctuations in populations of marine invertebrates, also doubts that the data collected and presented by Burkenroad were sufficient to justify the conclusion that definite periodic cycles in starfish population actually prevail.

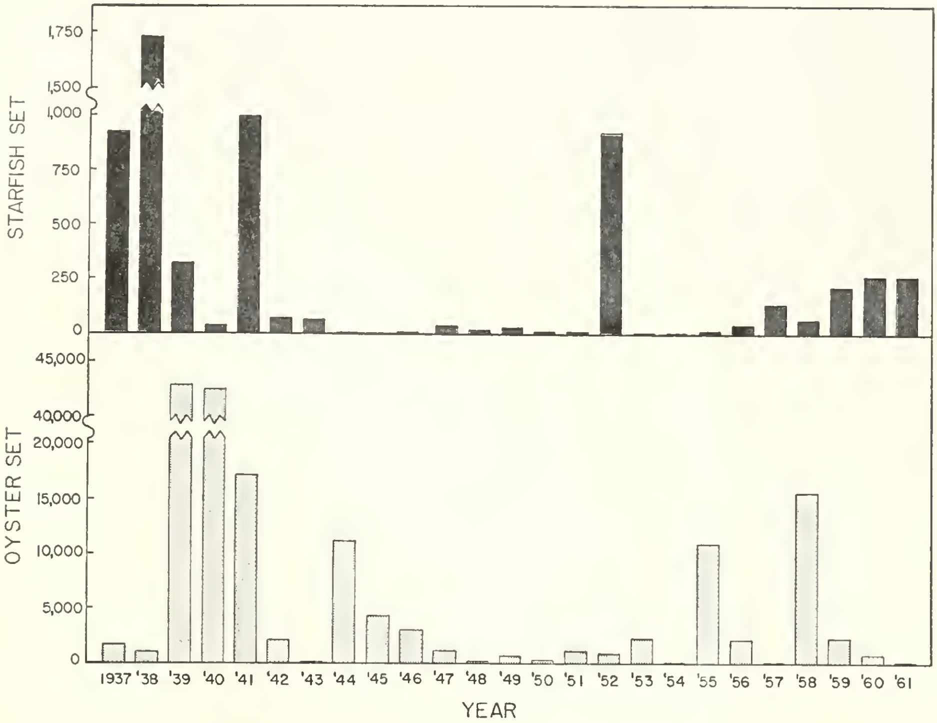


FIGURE 8. Average numbers of oyster and starfish set per station recorded in different years on 100 oyster shell-surfaces. Long Island Sound, 1937–1961.

Loosanoff *et al.* (1955) have shown that during the 18-year period, 1937–1954, no definite relationship was found between intensity of starfish and oyster setting in Long Island Sound. After carrying on these studies for an additional seven years, until 1961, the conclusion remained the same (Fig. 8). As in the past, we believe that the differences in intensity of setting of starfish and oyster larvae in the same years are principally due to causes of a specific nature, such as different food requirements of the larvae of the two animals and mortality caused by specific diseases and parasites of the larvae.

Still another factor to be considered in connection with mortality of larvae is the frequent presence in the water of certain external metabolites, the ectocrines, produced by many forms, including certain dinoflagellates, which may seriously affect the larval population of one species without affecting others. We hope that, because of recent advances in the technique of cultivation of eggs and larvae of marine invertebrates, which now permit conducting experiments on various aspects of behavior of these organisms and on the effects of natural and synthetic chemical compounds upon growth and survival of larval forms, many of these matters will be resolved in the near future.

During the quarter of a century when these studies were conducted many people contributed directly or indirectly to their success. I am grateful to them all, espe-

cially to my former aides, Messrs. Charles A. Nomejko, James B. Engle and John Malarkey, who spent countless hours examining the collectors and counting starfish set, and to Messrs. Joseph F. Lucash and Herman R. Glas, captains of our research boats, who, regardless of the weather, attended the stations in Long Island Sound to change and bring back the collectors, thereby making it possible to maintain these studies on a systematic basis. To Mrs. Susan Webb I am under obligation for preparing the illustrations, as well as for other help in completion of the manuscript, and to Miss Rita Riccio, Dr. James Hanks, and Mr. Harry C. Davis, for their helpful editorial suggestions. To Mrs. Barbara Myers I am obliged for the statistical analysis of the data.

SUMMARY

1. Setting of starfish (*A. forbesi*) was recorded at all depths of Long Island Sound from several feet above mean low water mark to 100 feet. No systematic observations were made at greater depth.

2. Starfish commence spawning when water temperature reaches approximately 15° C., which usually occurs about the middle of June. In some individuals spawning may continue into early September.

3. With the exception of 1945, when setting began and ended within the same day, September 11, the date of beginning of setting during the 25-year period varied from the last week of June to the last week of July.

4. The end of setting in the different years occurred between July 23 and October 1. In only 5 years setting continued into the second part of September.

5. The length of the setting period varied from one day, in 1945, to 91 days in 1961. In four years setting continued for 80 days or longer, but the average annual length of the setting period was about 52 days.

6. Intervals extending from the beginning of setting to the peak of the first or second waves of setting, and the time relationship between the first and second waves of setting did not show any orderly pattern.

7. During the 25-year period of observations the year 1938 held the highest rank in numbers of starfish set, while the year 1945 was the lowest. In general, the ranking indicated that there was no definite succession of good and poor years.

8. During the period of 17 years, 1940–1956, no appreciable relationship was found between numbers of adult starfish present during the pre-spawning period and intensity of starfish setting later in the season.

9. Our observations do not support the contention that peaks of the adult starfish population in Long Island Sound should have occurred in 1942 and in 1956.

10. No definite relationship was found between the intensity of starfish and oyster setting in Long Island Sound.

11. Larval diseases of specific nature, caused by various microorganisms, quality and quantity of food available, and the presence in the water of various ectocrines, may be principally responsible for the survival or mortality of starfish larvae and, therefore, for variations in intensity of setting of starfish in different years.

LITERATURE CITED

Agassiz, A., 1877. North American starfishes. *Mem. Museum Comparative Zoology,* **5**: 1–136

Burkenroad, M. D., 1946. Fluctuations in abundance of marine animals. *Science,* **103**: 684–686.

Clark, H. L., 1904. The echinoderms of the Woods Hole region. *Bull. U. S. Fish Comm. for 1902,* **22**: 545–576.

Coe, W. R., 1912. *State of Conn. Geological and Natural History Survey, Bull. No. 19,* pp. 1–152.

Coe, W. R., 1956. Fluctuations in populations of littoral marine invertebrates. *J. Mar. Res.,* **15**: 212–232.

Galtsoff, P. S., and V. L. Loosanoff, 1939. Natural history and method of controlling the starfish (*Asterias forbesi,* Desor). *Bull. U. S. Bur. Fish.,* **49**: 75–132.

Hancock, D. A., 1958. Notes on starfish on an Essex oyster bed. *J. Mar. Biol. Assoc.,* **37**: 565–589.

Hyman, Libbie Henrietta, 1955. The Invertebrates. Volume 4: Echinodermata. McGraw Hill Book Company, Inc., New York, 763 pp.

Loosanoff, V. L., 1936. Oyster pests control studies in Long Island Sound. *State of Conn. Biennial Rept. of the Shell-Fish Commissioners, 1935–36,* pp. 10–14.

Loosanoff, V. L., 1945. Effects of sea water of reduced salinities upon starfish, *A. forbesi,* of Long Island Sound. *Trans. Conn. Acad. Arts and Sciences,* **36**: 813–835.

Loosanoff, V. L., and H. C. Davis, 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull.,* **98**: 60–65.

Loosanoff, V. L., and H. C. Davis, 1963. Rearing of bivalve mollusks. Advances in Marine Biology, Vol. I, Academic Press, Inc., London and New York, pp. 1-136.

Loosanoff, V. L., and J. B. Engle, 1940. Spawning and setting of oysters in Long Island Sound in 1937, and discussion of the method for predicting the intensity and time of oyster setting. *Bull. U. S. Bur. Fish.,* **49**: 217–255.

Loosanoff, V. L., J. B. Engle and C. A. Nomejko, 1955. Differences in intensity of setting of oysters and starfish. *Biol. Bull.,* **109**: 75–81.

Loosanoff, V. L., and C. A. Nomejko, 1951. Spawning and setting of the American oyster, *O. virginica,* in relation to lunar phases. *Ecology,* **32**: 113–134.

Mead, A. D., 1901. The natural history of the starfish. *Bull. U. S. Fish Comm., 1899 (1901),* **19**: 203–224.

Riley, Gordon A., Shirley A. M. Conover, Georgiana B. Deevey, Robert J. Conover, Sarah B. Wheatland, Eugene Harris and Howard L. Sanders, 1956. Oceanography of Long Island Sound, 1952–1954. *Bull. Bingham Oceanogr. Coll.,* **15**: 1–414.

Smith, G. F. M., 1940. Factors limiting distribution and size in the starfish. *J. Fish. Res. Bd. Canada,* **5**: 84–103.

Vevers, H. G., 1949. The biology of *Asterias rubens* L.; growth and reproduction. *J. Mar. Biol. Assoc.,* **23**: 165–187.

UTILIZATION OF C^{14}-LABELED ACETATE AND GLYCEROL FOR LIPID SYNTHESIS DURING THE EARLY DEVELOPMENT OF SEA URCHIN EMBRYOS[1]

H. MOHRI[2]

Laboratory of Comparative Anatomy, the University of Palermo, Italy

Since lipids play significant roles in living organisms, not only as an energy source but also as constituents of cellular structures such as cell membrane or mitochondria and of some important enzymes such as cytochrome oxidase, it is worth examining lipid metabolism during the early embryonic development. Although considerable information is available concerning carbohydrate metabolism and protein metabolism at fertilization and during the development of sea urchins, little is known about their lipid metabolism. This is mainly due to the fact that the rapid advance in lipid chemistry, especially in methods of separating each class of lipids satisfactorily, has been achieved only recently with the development of silicic column chromatography and other techniques.

Accordingly, the classical works in this field have been restricted to measurements of the content of total lipid, total sterol or total phospholipid at different developmental stages (Ephrussi and Rapkine, 1928; Hayes, 1938). A more detailed analysis of lipids from sea urchin eggs and embryos was made by Öhman (1945), who tried to separate free and bound phospholipids, choline- and non-choline-containing phospholipids, and free and esterified cholesterol, and observed, for example, the conversion of cephalin from the free to the bound state immediately after fertilization, and its reversal during the ensuing development. In all of the above-mentioned works, a decline in the total lipid content was observed as development proceeded, suggesting the utilization of lipids as the energy source and/or as the precursor of some other compounds. A result of earlier determinations of respiratory quotient also indicated the combustion of lipids in the early phase of development (Öhman, 1940). None of these investigations, however, gave information as to lipid turnover or lipid synthesis during the early embryonic development, although Hayes (1938) reported a transient increase in the total lipid content after hatching, which was not observed by other authors.

Unfortunately, the studies so far made with radioactive isotopes on lipids of sea urchin eggs are very few in number. In spite of much work with radioactive phosphorus in sea urchin eggs, no data have been obtained on the uptake of the isotope in the lipid fraction of developing embryos, owing to the fact that the radioactivity found in the phospholipid fraction after the administration of P^{32} to

---

[1] Supported by grants from the Rockefeller Foundation and the National Institutes of Health, U. S. Public Health Service (RG 6211) to the Laboratory of Comparative Anatomy, the University of Palermo.

[2] On leave from the Biological Institute, College of General Education, University of Tokyo, Tokyo, Japan, as recipient of a scholarship from the Italian Government (1962-1963).

unfertilized and fertilized eggs was negligible as compared with the intense labeling of the acid-soluble fraction (Chambers, Whiteley, Chambers and Brooks, 1948). Hultin (1954), on the other hand, examined the metabolic utilization of C^{14}-labeled acetate in *Psammechinus miliaris* embryos, and observed that the incorporation of label from this substrate into fatty acids was highly intensified during the mesenchyme blastula stage, in parallel with the rapid increase of the incorporation into free hypoxanthine, protein amino acids and ribonucleic acid nucleotides. The experiment, however, included only the total fatty acid obtained after alkaline hydrolysis of the lipid extract.

In the present investigation, lipid metabolism during the early development of sea urchins was more closely studied, using acetate-1-C^{14} and glycerol-1-C^{14}. Lipids were extracted from eggs and embryos at different developmental stages after the administration of these isotopes, and were separated into several fractions by silicic acid column chromatography, followed by examination of the distribution of the label among these fractions. The total lipid content and the total phospholipid content were also estimated during the course of development. A preliminary account of this work has already been published (Mohri and Monroy, 1963).

Material and Methods

Eggs and embryos of the sea urchin, *Paracentrotus lividus,* were used in the present experiments. In applying labeled compounds, the method of Nakano and Monroy (1957), known to be successful with S^{35}-methionine, was tried first, *i.e.,* 25 μc. of the isotope were injected into the body cavity of the adult female. The eggs were collected four hours after the injection and were analyzed for the distribution of radioactivity among several fractions. In the present experiments, however, this method appeared to be unsuitable due to the very low uptake. Therefore, recourse was made to the usual method of incubating the embryos in sea water containing the labelled compounds.

The eggs, which were obtained by gently shaking the ovaries in sea water, were filtered through several layers of gauze and washed by low-speed centrifugation. A portion of the final egg suspension was taken out as the unfertilized sample and the rest was fertilized and allowed to develop in a large beaker, with continuous stirring, at about 18° C. At the desired stages, aliquots were taken from the synchronous suspension and eggs and embryos were collected by centrifugation. They were then re-suspended in 20 ml. of sea water to which 10–20 μc. of acetate-1-C^{14} or glycerol-1-C^{14} were added, and incubated for another two hours with gentle shaking. At the end of the incubation period, the eggs or embryos were again centrifuged down and washed three times with sea water. Unfertilized and newly fertilized eggs were deprived of their jelly-coat by treatment with acidified sea water. The sedimented eggs or embryos were homogenized in a chloroform-methanol (2:1) mixture, and the homogenate was filtered through a fat-free filter paper. The extract thus obtained was washed by adding 0.2 volume of distilled water containing an excess of non-radioactive substrate, according to the method of Folch *et al.* (1957). After separation into two layers in the cold, the upper methanol-water layer was discarded, and the lower chloroform layer was washed three more times with a small volume of distilled water containing an excess of non-radioactive substrate. A small portion of the final chloroform layer was used for the estimation of radioactivity and of the total lipids while the rest was dried out in a vacuum evaporator at 37° C. The dry residue was dissolved in a small volume of petroleum ether and subjected to fractionation through a silicic acid column, essentially as described by Mead and Fillerup (1954). Two and two-tenths grams of Mallinckrodt silicic acid were packed into a glass column and washed with 10 ml. each of methanol, chloroform, ether and petroleum ether in succession. The sample containing about 30 mg. of lipids was then placed on top of the column and eluted first with 20 ml. each of petroleum ether (hydrocarbons); petroleum ether-ether 99:1 (cholesterol esters); petroleum ether-ether 9:1 (triglycerides + free fatty acids); petroleum ether-ether 3:1 (free cholesterol); petroleum ether-ether 1:1 (diglycrides); ether (monoglycerides). This was followed by an elution with 25 ml. each of chloroform-methanol 4:1 (cephalin); chloroform-methanol 3:2 (inositol phospholipid + lecithin); chloroform-methanol 1:4 (lecithin + sphingomyelin); methanol (lysolecithin).

For the measurements of radioactivity, lipid samples were placed in the counting vials, dried, weighed and counted on an EKCO scintillation counter, as previously reported from this laboratory (Giudice, Vittorelli and Monroy, 1962).

The one-dimensional ascending chromatography of phospholipids on silicic acid-impregnated paper was carried out by using chloroform-methanol 4:1 as a solvent (Lea *et al.*, 1955). The lipid spots were detected by staining with 0.001% Rhodamine 6G.

The estimation of total lipids, total phospholipids and total nitrogen was made as follows. Aliquots of eggs or embryos at the desired stage were collected by gentle centrifugation and homogenizated with 6% perchloric acid. A small portion of the homogenate was used for the determination of total nitrogen. The larger portion of the homogenate was centrifuged and the supernatant was discarded. The residue was re-extracted with 6% perchloric acid, followed by washing with distilled water. The residue was then extracted twice with ethanol and once with boiling ethanol-ether (3:1). The extracts were combined and evaporated to dryness. The dry residue was extracted with petroleum ether, filtered through a sintered glass filter and used for the determination of total lipid and total phospholipid. For the total lipids, the extract was evaporated again and the residue was weighed. For the total phospholipids, phosphorus content of the extract was estimated and multiplied by 25, assuming that phospholipids contain 4% phosphorus.

The samples for the determination of total nitrogen were combusted and the nitrogen content was estimated by direct Nesslerization. Total phosphorus was determined according to the method of Allen (1940), using ammonium molybdate and amidol reagents.

RESULTS

Changes in total lipid, phospholipids and total nitrogen during development

As a first step in these studies on lipid metabolism of sea urchin embryos, the changes in the amounts of total lipids and of phospholipids were followed throughout the early development up to the pluteus stage, together with the change in total nitrogen content. As can be seen from Figure 1, the content of total lipids in unfertilized eggs was 2.68 mg./10^5 eggs. Since the total nitrogen in the unfertilized eggs amounted to 1.61 mg./10^5 eggs, the above value corresponds to 166 mg./100 mg. total nitrogen and is consistent with the figures of 144 or 178 mg./100 mg. total nitrogen calculated by Öhman (1945) from the data obtained by Ephrussi and Rapkine (1928) with *Paracentrotus lividus,* and of 174.2 or 202.8 mg./100 mg. total nitrogen obtained by Öhman (1945) with *Echinocardium cordatum.* The total lipid content showed essentially no change until the mesenchyme blastula stage, after which it decreased considerably toward the pluteus stage. The quantity of phospholipids changed in a very similar manner to that of total lipid, occupying about one-third of the total lipid throughout the early development. Phospholipids of *E. cordatum* eggs have also been reported to be around one-third of the total lipid (Öhman, 1945).

The total nitrogen, on the other hand, decreased slightly but steadily until the gastrula stage and then showed an increase. Such a decrease during the first 24 hours of development of *P. lividus* has been reported by Nakano and Monroy (1958), although Gustafson and Hasselberg (1951) reported a constancy in total nitrogen content until at least the early pluteus stage.

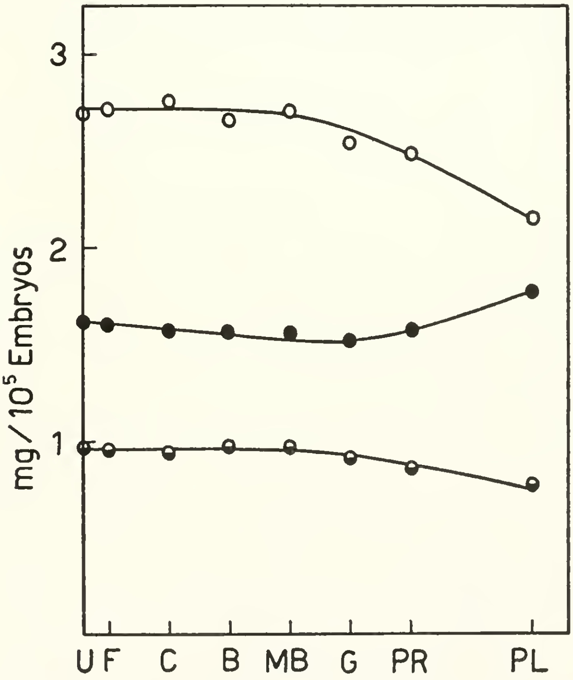


FIGURE 1. Changes in the content of total lipid (○), phospholipids (◒) and total nitrogen (●) during the early development of the sea urchin, *Paracentrotus lividus.* U: unfertilized egg; F: newly fertilized egg; C: cleavage stage (32–64 cells); B: blastula; MB: mesenchyme blastula; G: gastrula; PR: prism stage; PL: pluteus.

Incorporation of acetate-1-C^{14} into total lipids

Figure 2 shows the relationship between the time of incubation with acetate-1-C^{14} (10 μc.) and the uptake of the isotope in the total lipids of early gastrulae. A linear increase of the incorporation was observed at least during the first 1.5 hours, after which the rate of incorporation declined. The standard incubation time of two hours which was used in the following experiments is thus located at about the end of the linear increase.

The changes in the rate of incorporation of acetate-1-C^{14} into the total lipids in the course of development are shown in Figure 3. In this experiment, eggs or embryos were incubated with 20 μc. of acetate-1-C^{14}. Very slight radioactivity was found in the total lipids of unfertilized eggs. The activity increased somewhat after fertilization. A rapid rise in the rate of incorporation of the isotope then took place with progressive development and continued until at least the prism stage, after which it began to decrease.

As described above, no significant change was found in the content of total lipid until at least the mesenchyme blastula stage and a decline in content occurred after this stage. In the hope of finding out what the steep increase in incorporation in the total lipids reflects, the following experiment was carried out. The eggs were allowed to develop to the blastula stage; the embryos were washed and incubated with 10 μc. of acetate-1-C^{14} for two hours. The sample was then divided into two equal portions, one of which was immediately processed for the extrac-

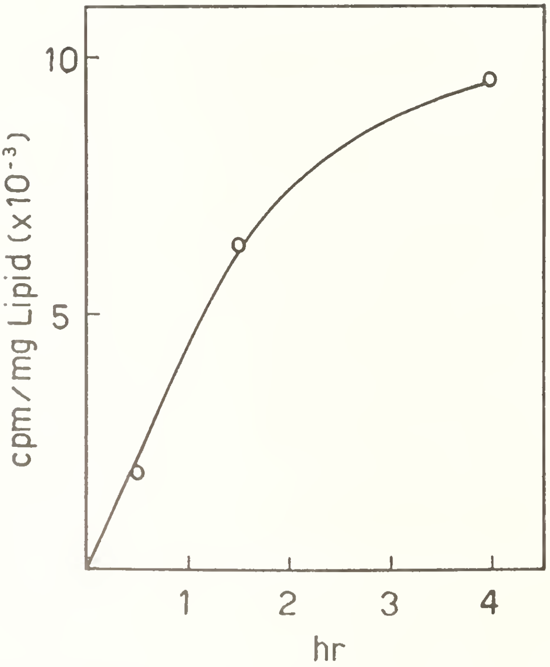


FIGURE 2. Relationship between the incubation time and the incorporation of acetate-1-C^{14} into the total lipids. Early gastrula. Temperature, 18° C.

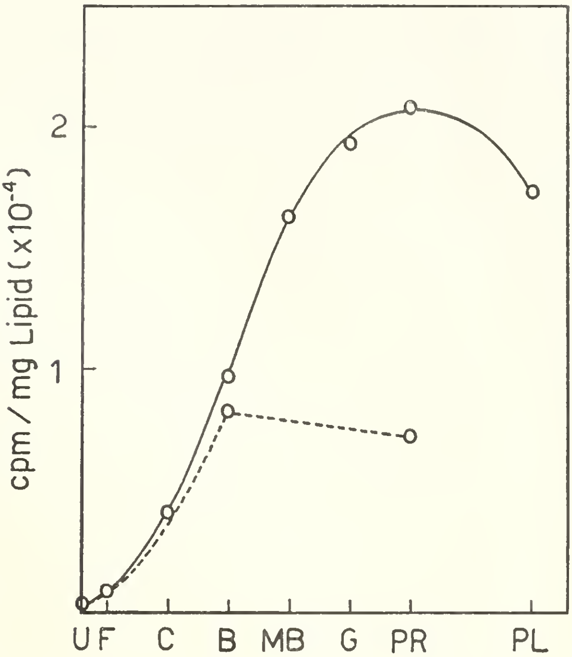


FIGURE 3. Incorporation of acetate-1-C^{14} into total lipids of sea urchin embryos during the early development (solid line). For explanation of dotted line, see text. Abbreviations as in Figure 1.

tion of the total lipids as described above. The other portion was centrifuged, washed three times with fresh sea water and re-suspended in sea water containing an excess of C^{12}-acetate. The volume of the final suspension was adjusted to that of the original suspension before the incubation with the isotope, and the suspension was cultured further with constant stirring. When the prism stage was reached after about 24 hours, the embryos were collected and subjected to the extraction procedure. As shown by the dotted line in Figure 3, only a slight decrease in the specific activity of total lipids occurred during the period of development. Since, however, the content of total lipids also somewhat decreased by the prism stage, the slight reduction in the specific activity corresponds to approximately a 20% loss of total radioactivity found in the total lipids of the blastulae. This might be partly due to the breakdown of lipids which took place after the mesenchyme blastula stage and partly due to the replacement of C^{14} by C^{12} which came from both the culture medium and the metabolic pool.

In the same experiment, the total lipids were further separated into an acetone-soluble (non-phospholipids) and an acetone-insoluble (phospholipids) fraction. At the blastula stage, 62.6% of the radioactivity of total lipids was found in the acetone-soluble fraction and 37.4% in the acetone-insoluble fraction, while at the prism stage, 54.8% was recovered from the former fraction and 45.2% from the latter. If we assume that no interconversion took place between the two fractions

TABLE I

Fractionation of the total lipid from early gastrula of sea urchin, Paracentrotus lividus, through silicic acid column

Fr. No.	Solvents	Yield mg.	Radioactivity c.p.m. (c.p.m./mg. lipid)
1	Petroleum ether	2.1	1,150 (550)
2	99% P.e. + 1% Ether	1.3	1,360 (1,050)
3	90% P.e. + 10% Ether	15.9	43,000 (2,700)
4	75% P.e. + 25% Ether	3.35	5,200) (1,500)
5	50% P.e. + 50% Ether	1.4	3,200 (2,300)
6	Ether	3.75	2,700 (720)
7	80% $CHCl_3$ + 20% MeOH	4.05	136,000 (34,000)
8	60% $CHCl_3$ + 40% MeOH	2.4	14,200 (5,900)
9	20% $CHCl_3$ + 80% MeOH	3.93	15,300 (3,900)
10	MeOH	0.09	500 (5,600)

during development and take into account the 20% loss in the total radioactivity of total lipids, then these figures imply that the label once incorporated in phospholipids at the blastula stage was not lost during subsequent development, only the label in non-phospholipids disappearing. It appears, therefore, that the phospholipids newly synthesized at the blastula stage were not utilized during the later phase of development in spite of the decrease in the content of phospholipids after the mesenchyme blastula stage. Another alternative, however, is also possible, since it has been reported that the synthesis of phospholipids proceeds *via* diglycerides (*cf.* Kennedy, 1957).

After hydrolysis of the total lipids with alcoholic potassium hydroxide, more than 95% of total radioactivity was recovered in the saponifiable fraction. Since cholesterol forms only a small percentage of the total lipids (see below), most of the C^{14} from acetate-1-C^{14} should be incorporated into the fatty acid moieties of glycerides and of phospholipids.

Distribution of C^{14} in the lipid fractions after administration of acetate-1-C^{14}

The total lipids were further fractionated on the silicic acid column as described above and the distribution of radioactivity among the fractions was estimated. Table I shows a typical result obtained with the total lipids from early gastrulae.

As to the relative amounts of the various fractions, the most predominant one is fraction 3 (triglycerides + little free fatty acids), occupying 41.5% of the total lipid. Only fraction 4 was shown to contain an appreciable amount of cholesterol by the Liebermann-Burchard reaction. This fraction amounted to 8.8% of the total lipid, and thus appeared to consist mainly of free cholesterol. Only a weak reaction was obtained with fraction 2, which would be expected to contain cholesterol esters. Actually, the content of esterified cholesterol in the eggs of *E. cordatum* had already been reported to be only 7.5% of the total cholesterol content (Öhman, 1945), which is consistent with the present result. Samples from fractions 7 to 10, eluted by mixtures of chloroform and methanol, were all found to contain phosphorus and should therefore be phospholipids. Since small amounts of silicic acid leaked out of the column with these fractions, in spite of previous washing of the column with chloroform and methanol, the yields of these fractions

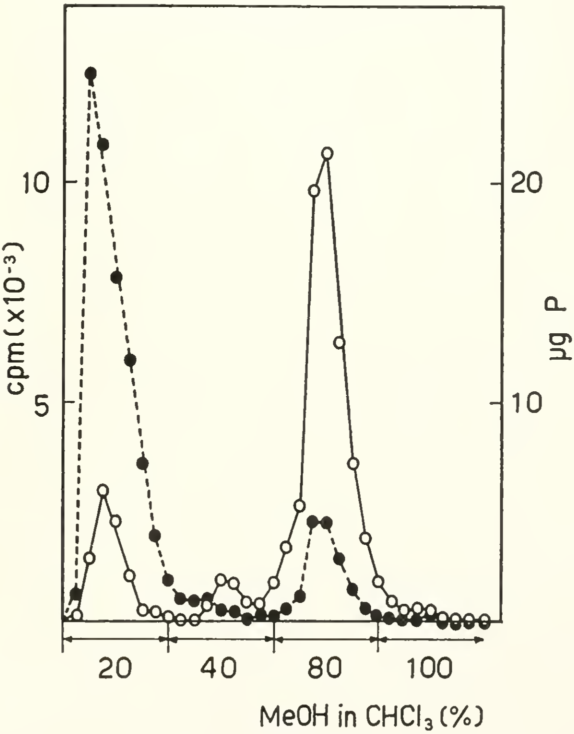


FIGURE 4. Chromatogram of combined phospholipid fractions obtained by the fractionation of total lipid from early gastrulae incubated with acetate-1-C^{14}. Open circle and solid line indicates phosphorus content of each fraction, and solid circle and dotted line indicates total radioactivity of each fraction.

TABLE II

Incorporation of acetate-1-C^{14} into lipid fractions of sea urchin embryos, Paracentrotus lividus. Numbers with and without parentheses refer to specific radioactivity (c.p.m./mg. lipid) and total radioactivity (c.p.m.), respectively. Abbreviation of stages as in Figure 1

Stage Fr. No.	U	F	C	B	MB	G	PR	PL
1, 2	100 (50)	200 (200)	8,500 (12,500)	2,900 (4,100)	2,200 (2,600)	4,800 (6,300)	6,200 (20,500)	10,200 (17,100)
3	1,600 (100)	3,200 (200)	12,800 (800)	70,000 (5,200)	100,000 (7,800)	111,000 (8,000)	127,000 (8,500)	57,000 (4,100)
4	300 (90)	400 (100)	23,000 (6,600)	28,000 (10,500)	29,000 (15,000)	29,000 (12,700)	37,000 (9,900)	30,000 (7,400)
5	200 (80)	50 (70)	4,300 (3,500)	8,900 (7,100)	14,000 (13,300)	10,900 (11,200)	7,600 (14,400)	1,800 (4,300)
6	100 (100)	200 (300)	2,600) (3,700)	4,000 (5,600)	5,200 (23,000)	8,700 (16,600)	5,200 (23,000)	8,200 (47,000)
7	2,900 (600)	16,700 (3,500)	66,000 (11,500)	100,000 (21,000)	170,000 (38,000)	270,000 (57,000)	330,000 (63,000)	360,000 (66,000)
8	700 (400)	1,900 (900)	12,000 (3,300)	36,000 (12,700)	13,000 (18,400)	67,000 (25,000)	85,000 (33,000)	62,000 (24,000)
9	1,100 (200)	4,700 (700)	13,500 (2,400)	19,100 (4,100)	29,000 (6,600)	34,000 (6,900)	63,000 (13,400)	42,000 (9,700)
10	50 (300)	200 (800)	300 (3,500)	400 (4,200)	900 (11,800)	900 (7,000)	3,000 (13,200)	5,900 (17,300)

were calculated from the phosphorus figures, assuming that phospholipids contain 4% phosphorus. The amount of phospholipids thus calculated was 27.4% of the total lipids. No attempts were made to examine further the nature of other fractions because of their limited quantities and relatively low radioactivity.

Most of the radioactivity was found in the triglyceride and phospholipid fractions, especially in fraction 7 which in this case was found to contain about 60% of the total radioactivity and to have the highest specific activity. As will be described below, this fraction seems to be the cephalin. Other phospholipid fractions also showed relatively high specific activities. The triglyceride fraction, on the other hand, had rather low specific activity.

The phospholipid fractions obtained here were again combined and rechromatographed on silicic acid column, with a view to obtaining more precise results about the distribution of C^{14} among phospholipids. The same solvent system was used for elution, *i.e.*, chloroform-methanol 4:1, 3:2, 1:4 and methanol. The eluates were collected in 3-ml. fractions, and the radioactivity and phosphorus content of each fraction were estimated. The results are presented in Figure 4. At least four distinct peaks were obtained. The first peak (eluted by chloroform-

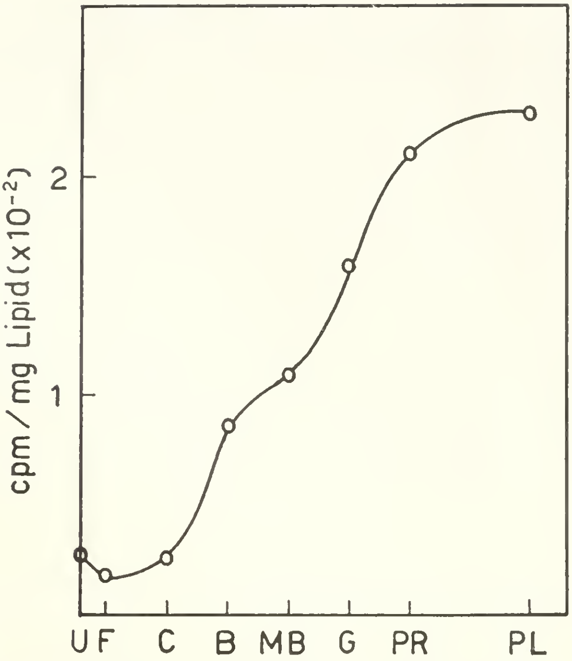


FIGURE 5. Incorporation of glycerol-1-C^{14} into the total lipid of sea urchin embryos during the early development. Abbreviations as in Figure 1.

methanol 4:1) contained much radioactivity. When a sample of this peak was chromatographed on silicic acid-impregnated paper, the spot of phosphatidylethanolamine was obtained. The third peak (eluted by chloroform-methanol 1:4) was the largest, but contained much less radioactivity. Paper chromatography showed that this peak contained lecithin. The second and the fourth peaks, which appeared to be inositol phospholipids and lysolecithin, respectively, were the minor components, and not much radioactivity was located in them. Thus, it appeared very clearly that the cephalin fraction was labeled with acetate-1-C^{14} much more strongly than the lecithin and other fractions.

The results obtained with several developmental stages are summarized in Table II. The same samples as those in Figure 3 were fractionated through silicic acid columns. Since, especially with lipids obtained from the embryos at later developmental stages, fraction 1 was sometimes followed by the elution of triglycerides and it was impossible to separate fraction 1 from fraction 2, these two fractions were combined in this table. On the whole, no great change was observed in the distribution pattern of C^{14} among lipid fractions during embryonic development, the increase in the labeling of almost all fractions being parallel to the rise in the incorporation into the total lipids. In this experiment, too, fraction 7 was most strongly labeled with acetate-1-C^{14} throughout development, as indicated by both the total and the specific activities. The total incorporation into the triglyceride fraction was again found to be high; the specific activity was, however, rather low.

It is apparent that the reduction in the isotope incorporation into the total lipid observed at the pluteus stage was mainly due to the decreased rate of incorporation into this fraction. Among the other fractions, the labeling of fraction 8 was relatively high.

Incorporation of glycerol-1-C^{14} into total lipids and lipid fractions

Similar experiments were carried out with glycerol-1-C^{14}. Figure 5 indicates the incorporation of glycerol-1-C^{14} into the total lipids at different stages of development. In this case, eggs or embryos were incubated with 10 μc. of the isotope. Apart from the much lower labeling of the total lipids (as can be seen from a comparison of the unit on the ordinate in Figure 3 and Figure 5) the general pattern was not very different from that obtained with acetate-1-C^{14}. However, the rate of incorporation dropped somewhat after fertilization and showed a slowing down during the blastula stage. The maximum was then reached at the pluteus instead of the prism stage. After alkaline hydrolysis of the total lipids, about half of the total radioactivity was found in the saponifiable fraction. This

TABLE III

Incorporation of glycerol-1-C^{14} into lipid fractions of sea urchin embryos, Paracentrotus lividus. Numbers with and without parentheses refer to specific radioactivity (c.p.m./mg. lipid) and total radioactivity (c.p.m.), respectively. Abbreviation of stages as in Figure 1

Stage Fr. No.	U	F	C	B	MB	G	PR	PL
1, 2	0 (0)	0 (0)	66 (85)	38 (31)	32 (40)	48 (93)	53 (85)	0 (0)
3	162 (10)	34 (2)	89 (4)	530 (29)	880 (53)	740 (45)	1140 (74)	780 (44)
4	3 (1)	0 (0)	0 (0)	92 (23)	71 (25)	44) (18)	57 (32)	101 (34)
5	53 (27)	10 (9)	12 (13)	29 (158)	38 (45)	77 (79)	83 (82)	18 (44)
6	0 (0)	8 (27)	3 (5)	41 (60)	23 (55)	12 (38)	0 (0)	35 (200)
7	220 (66)	310 (91)	370 (92)	910 (240)	990 (290)	2700 (620)	3500 (910)	6100 (1190)
8	210 (159)	155 (129)	380 (113)	890 (260)	930 (330)	1000 (420)	1210 (970)	900 (390)
9	100 (16)	88 (15)	210 (15)	350 (89)	530 (109)	740 (166)	850 (188)	830 (210)
10	0 (0)	0 (0)	9 (95)	68 (1100)	22 (280)	41 (380)	81 (390)	62 (220)

result seems to indicate that some portion of glycerol was metabolized before entering the lipids.

The distribution of radioactive carbon among lipid fractions after the administration of glycerol-1-C^{14} is shown in Table III. No essential difference was found between the results obtained with C^{14}-labeled acetate and those with C^{14}-labeled glycerol, except that the labeling of the lipid fractions occurred to a much lesser extent in the latter case. The most intense uptake of the label was found in the phospholipid fractions, especially in fractions 7 and 8, while the labeling of fraction 3 (triglyceride) was not as strong, judging from its specific activity.

DISCUSSION

The present estimations of total lipid content and of phospholipid content at different stages in sea urchin development indicate that the disappearance of lipids does not occur until the mesenchyme blastula stage, although a considerable decrease is observed during gastrulation and subsequent development. The results are thus somewhat different from those obtained with *Arbacia punctulata* by Hayes (1938), who reported a sudden drop in the total lipids immediately after fertilization and a rise after hatching, which was again followed by a steady decrease toward the final stages observed (43 hours, 10 minutes after fertilization). He also reported fluctuations in the content of phospholipids during early development. No change in lipid content at fertilization or during the first 5 hours of development was obtained, however, by Parpart (1941) with the same species, *A. punctulata*. Furthermore, a recent analysis of phosphorus compounds during the early development of the Japanese sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, produced the same result as that reported here concerning the change in the phospholipid content (Yamagami, 1963). Whether the difference in comparison with Hayes' results is due to species difference or to some technical reason is not clear. The present author is, however, inclined towards the latter alternative.

With regard to the main source of energy during the early embryonic development of the sea urchin, many determinations of the respiratory quotient (R.Q.) have been made. According to the latest results obtained by Isono (1963) with eggs and embryos of *Anthocidaris crassispina*, the R.Q. remains constant (near to unity) from the early stages up to hatching, suggesting that the oxidative breakdown of carbohydrates is prevalent during this phase. From the swimming blastula stage, the R.Q. begins to decrease, indicating that in the later phase of development combustion of lipids may be the main source of energy. Isono's results are thus consistent with the present finding that the total lipid content diminishes only after the mesenchyme blastula stage. On the other hand, Öhman (1940), working on the same material as used here, obtained a R.Q. of 0.73 during one and two hours after fertilization and 0.85 during 7 and 8 hours after fertilization. This, together with his observation that there was a slight reduction in the total lipids until 7 hours after fertilization (Öhman, 1945), led him to suggest that the basal respiration consists of fat combustion, while the increasing part of respiration during blastula formation is supplemented by the oxidation of carbohydrates. However, in the present experiments the decrease in the total lipid content in the early phase of development has been found to be negligible. This is consistent with the R.Q. values near to or higher than 0.9 during these early stages (*cf.* Isono, 1963). Furthermore, available evidence indicates a reduction in the glycogen content after fertilization (Örström and Lindberg, 1940; Monroy and Vittorelli, 1960) and the simultaneous activation of the pentose phosphate cycle (Aketa, Bianchetti, Marré and Monroy, in press). The activity of this cycle increases during the early stages of development (Krahl, 1956; Bäckström, Hultin and Hultin, 1960). This, however, does not exclude the possibility of the participation of lipids as a minor energy source in the early phase of development. It seems likely, therefore, that in the early stages of sea urchin development respiration proceeds mainly through the oxidation of carbohydrates, possibly *via* the pentose phosphate cycle, and then, after the mesenchyme blastula stage, through the oxidation of lipids.

The administration of radioactive acetate and glycerol resulted in only slight labeling of lipids in unfertilized eggs, indicating that the eggs do not metabolize lipids to any appreciable extent if they remain unfertilized. After fertilization, however, a rapid increase in isotope incorporation occurs with progressive development. The label from acetate-1-C^{14} is almost all incorporated into fatty acid moieties of the lipids. It is interesting to note that in unfertilized eggs the activity of acetyl thiokinase, the enzyme catalyzing the first step in the synthesis of long-chain fatty acids from acetate, has been found to be as active as in fertilized eggs and in blastulae (Maggio, personal communication). It would appear that, at least in the early stages of development, the increase observed in the incorporation of acetate-1-C^{14} into lipids is not due to an increase in the amount of this enzyme, but rather to a structural and physiological rearrangement of cellular components which would allow the enzyme to react with its substrate. Alternatively, it may be due to the activation of other enzymes participating in the synthesis of fatty acids, which in the unfertilized egg may be limiting. The relatively low incorporation of glycerol-1-C^{14} into lipids as compared with that of acetate-1-C^{14} might be explained by the fact that α-glycerophosphate, but not free glycerol, is the direct substrate for the synthesis of glycerides and phospholipids (*cf.* Kennedy, 1957). Evidence showing the breakdown of glycerol before entering lipids has also been obtained.

It has been found that high specific activity is always associated with phospholipid fractions, among which the cephalin fraction is the most strongly labeled, while the labeling of lecithin is not so extensive. In this respect, it is noteworthy that during the early development of rainbow trout the lecithin content is much reduced after hatching, thus accounting for the disappearance of all the phospholipids during the later phase of development, whereas there is no significant change in the cephalin content (Yamagami and Mohri, 1962). These results seem to suggest that lecithin is preferentially utilized by the embryonic tissues as an energy source or precursor of other compounds. If this is also true in sea urchin embryos, although no efforts were made to separate cephalin and lecithin quantitatively in the present experiments, the relatively low incorporation into lecithin could be explained by the preponderance of the degradation over the synthetic processes of this compound. In fact, Öhman (1945) reported an increase in the cephalin content concomitant with a decrease in the lecithin content during development. A more detailed analysis is desirable to make this point clear.

Hultin (1954) obtained a similar increase in the rate of incorporation of acetate-1-C^{14} into the total fatty acid during the early development of *Psammechinus* embryos. In this case, however, the incorporation was not very extensive before the mesenchyme blastula stage and a sharp increase occurred only after this stage. His results thus coincide fairly well with the observation that the activation of many mitochondrial enzymes occurs at the beginning of mesenchyme blastula (Gustafson and Hasselberg, 1951). In the present experiments, on the other hand, the rapid increase was seen to start soon after fertilization, especially with acetate as substrate, and the incorporation curve seems to have more similarity to the population curve of mitochondria obtained by Gustafson and Lenicque (1952, 1955) than to the curve of the activity of mitochondrial enzymes. The discrepancy in comparison with Hultin's results could be due to species difference. According to Gustafson and Lenicque (1952, 1955), the number of mitochondria first increases during the early blastula stage, then remains constant until the mesenchyme blastula stage, and shows a second rise after this stage, thus corresponding to the curve of oxygen uptake (Lindahl, 1939). Such a two-step increase in the course of development has been observed in several instances with regard to the incorporation of labeled substrates, *e.g.*, the incorporation of C^{14}-labeled acetate into free hypoxanthine and ribonucleic acid nucleotides (Hultin, 1954), the uptake of C^{14}-labeled glucose by the acid-soluble and the total protein fraction (Monroy and Vittorelli, 1962), and the incorporation of labeled amino acids into total embryo and proteins of subcellular fractions (Giudice, Vittorelli and Monroy, 1962). The present results obtained with glycerol-1-C^{14} also suggest the presence of a plateau in the incorporation curve from the blastula to the mesenchyme blastula stage, although this is not the case with acetate-1-C^{14}.

The rapid uptake of the isotopes into lipids takes place without an increase in the total lipid content and even with a decrease in this content after the mesenchyme blastula stage. The present finding, that the label once incorporated into phospholipids at an early stage is hardly lost during subsequent development, suggests that the syntheses of some components are essential for, but not utilized in, the later phase of development. The lipids disappearing at the later stages appear to be somewhat different in nature or in localization from the newly synthesized ones. Since the incorporation is also especially high into phospholipids, which are known to be an important constituent of membranous structures, it is highly probable that the lipids synthesized during development are associated with membrane proliferation. Although the above-mentioned similarity between the incorporation curve and the curve of mitochondrial population suggests that the active lipid synthesis takes place in the mitochondrial membrane, it is also conceivable that the cell surface with its tremendous increase as a result of cell divisions may be an important element in lipid synthesis. This remains, however, an open problem.

The author wishes to express his cordial thanks to Professor A. Monroy for his many valuable suggestions and kind hospitality, both of which made this experiment possible. The technical assistance of Mr. O. A. Oliva is also acknowledged. Finally, thanks are due to Miss J. Baxandall for her kind help in preparing the manuscript.

SUMMARY

1. Lipid metabolism during the early embryonic development of the sea urchin, *Paracentrotus lividus*, was studied, using acetate-1-C^{14} and glycerol-1-C^{14} as precursors.

2. The content of total lipids per embryo showed no significant change up to the mesenchyme blastula stage but began to decrease considerably with the onset of gastrulation. Phospholipids, which account for about one-third of the total lipids, showed the same change as the total lipids. Total nitrogen, on the other hand, continued to decrease slightly until the gastrula stage and then began to increase.

3. The total lipids were fractionated through a silicic acid column. The predominant components were found to be triglycerides and phospholipids. For example, the figures obtained with lipids extracted from early gastrulae were 41.5% of triglycerides, 27.4% of phospholipids and 8.8% of free cholesterol. Only small amounts of cholesterol esters and free fatty acids were found.

4. Acetate-1-C^{14} was only slightly incorporated into the total lipids of unfertilized eggs. The incorporation, however, increased very rapidly in an S-shaped curve with progressive development, reaching a maximum at the prism stage. The incorporation rate was somewhat reduced thereafter. Most of the C^{14} was found to be incorporated into fatty acid moieties.

5. After the administration of acetate-1-C^{14}, much of the C^{14} was incorporated into the phospholipid and triglyceride fractions throughout the early embryonic development. The highest specific activity was always obtained with the cephalin fraction, the lecithin fraction having much lower specific activity. The specific activity of the triglyceride fraction, on the other hand, was fairly low as compared with those of other fractions.

6. Similar results were obtained with glycerol-1-C^{14}, except that the incorporation of this precursor occurred to a much lesser extent than that of acetate-1-C^{14}. In this case, however, the incorporation into the total lipids decreased somewhat after fertilization and reached the maximum at the pluteus stage, with a shoulder during the blastula stage.

7. The highest specific activity was also found in the cephalin fraction after the administration of glycerol-1-C^{14}. As a considerable amount of C^{14} was found in the saponifiable fraction as well as in the unsaponifiable fraction, some glycerol seems to be metabolized before being incorporated into lipids.

8. The above results indicate that lipid metabolism is much intensified during the embryonic development of the sea urchin, especially in its later phase. As C^{14}, once taken up into phospholipids at the blastula stage, was not lost in any significant amount during the subsequent development to the prism stage, it seems likely that new synthesis occurs in some lipid components, especially in phospholipids, which are essential for subsequent development.

LITERATURE CITED

AKETA, K., R. BIANCHETTI, E. MARRÉ AND A. MONROY, 1964. Hexose monophosphate level as a limiting factor for respiration in unfertilized sea urchin eggs. *Biochem. Biophys. Acta,* in press.

ALLEN, R. J. L., 1940. The estimation of phosphorus. *Biochem. J.*, **34**: 858–865.

BÄCKSTRÖM, S., K. HULTIN AND T. HULTIN, 1960. Pathway of glucose metabolism in early sea urchin development. *Exp. Cell Res.*, **19**: 634–636.

CHAMBERS, E. L., A. WHITELEY, R. CHAMBERS AND S. C. BROOKS, 1948. Distribution of radioactive phosphate in the eggs of the sea urchin *Lytechinus pictus*. *Biol. Bull.*, **95**: 263.

EPHRUSSI, B., AND L. RAPKINE, 1928. Composition chimique de l'oeuf d'oursin *Paracentrotus lividus* Lk. et ses variations au cours du développement. *Ann. de Phys. et de Phys.-chim. Biol.*, **4**: 386–398.

FOLCH, J., M. LEES AND G. H. SLOANE-STANLEY, 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226**: 497–509.

GIUDICE, G., M. L. VITTORELLI AND A. MONROY, 1962. Investigations on the protein metabolism during the early development of the sea urchin. *Acta Embryol. Morphol. Exp.*, **5**: 113–122.

GUSTAFSON, T., AND I. HASSELBERG, 1951. Studies on enzymes in the developing sea urchin egg. *Exp. Cell Res.*, **2**: 642–672.

GUSTAFSON, T., AND P. LENICQUE, 1952. Studies on mitochondria in the developing sea urchin egg. *Exp. Cell Res.*, **3**: 251–274.

GUSTAFSON, T., AND P. LENICQUE, 1955. Studies on mitochondria in early cleavage stages of the sea urchin egg. *Exp. Cell Res.*, **8**: 114–117.

HAYES, F. R., 1938. The relation of fat changes to the general chemical embryology of the sea urchin. *Biol. Bull.*, **74**: 267–277.

HULTIN, T., 1954. Incorporation of C^{14}-labeled carbonate and acetate into sea urchin embryos. *Ark. Kemi*, **6**: 195–200.

ISONO, N., 1963. Carbohydrate metabolism in sea urchin eggs. III. Changes in respiratory quotient during early embryonic development. *Annot. Zool. Japon.*, **36**: 126–132.

KENNEDY, E. P., 1957. Metabolism of lipids. *Ann. Rev. Biochem.*, **26**: 119–148.

KRAHL, M. E., 1956. Oxidative pathways for glucose in eggs of the sea urchin. *Biochem. Biophys. Acta*, **20**: 27–32.

LEA, C. H., D. N. RHODES AND R. D. STOLL, 1955. Phospholipids. 3. On the chromatographic separation of glycerophospholipids. *Biochem. J.*, **60**: 353–363.

LINDAHL, P. E., 1939. Zur Kenntnis der Entwicklungsphysiologie des Seeigeleies. *Zeitschr. vergl. Physiol.*, **27**: 233–250.

MEAD, J. F., AND D. L. FILLERUP, 1954. Plasma lipids in fat deficiency. *Proc. Soc. Exp. Biol. Med.*, **86**: 449–451.

MOHRI, H., AND A. MONROY, 1963. Lipid metabolism of sea urchin embryos. *Zool. Mag.*, **72**: 265–266.

MONROY, A., AND M. L. VITTORELLI, 1960. On a glycoprotein of the sea urchin eggs and its changes following fertilization. *Experientia*, **16**: 56.

MONROY, A., AND M. L. VITTORELLI, 1962. Utilization of C^{14}-glucose for amino acids and protein synthesis by the sea urchin embryo. *J. Cell. Comp. Physiol.*, **60**: 285–288.

NAKANO, E., AND A. MONROY, 1957. A method for the incorporation of radioactive isotopes in the sea urchin egg. *Experientia*, **13**: 416.

NAKANO, E., AND A. MONROY, 1958. Incorporation of S^{35}-methionine in the cell fractions of sea urchin eggs and embryos. *Exp. Cell Res.*, **14**: 236–244.

ÖHMAN, L. O., 1940. Über die Veränderung des respiratorischen Quotienten während der Frühentwicklung des Seeigeleies. *Ark. Zool.*, **32A**: Nr.15.

ÖHMAN, L. O., 1945. On the lipids of the sea urchin egg. *Ark. Zool.*, **36A**: Nr.7.

ÖRSTRÖM, Å., AND O. LINDBERG, 1940. Über den Kohlenhydratstoffwechsel bei der Befruchtung des Seeigeleies. *Enzymologia*, **8**: 367–384.

PARPART, A. K., 1941. Lipoprotein complexes in the egg of *Arbacia*. *Biol. Bull.*, **81**: 296.

YAMAGAMI, K., 1963. Changes in the amounts of some endogenous phosphates in the development of the sea urchin. *Zool. Mag.*, **72**: 252–255.

YAMAGAMI, K., AND H. MOHRI, 1962. Phosphorus metabolism in fish eggs. IV. Column chromatographic separation of phospholipids in the rainbow trout during embryonic and postembryonic development. *Sci. Pap. Coll. Gen. Educ., Univ. Tokyo*, **12**: 233–240.

UPTAKE OF RADIOACTIVE GLUCOSE AND AMINO ACIDS AND THEIR UTILIZATION FOR INCORPORATION INTO PROTEINS DURING MATURATION AND FERTILIZATION OF THE EGGS OF ASTERIAS FORBESII AND SPISULA SOLIDISSIMA[1]

ALBERTO MONROY[2] AND HELEN TOLIS[3]

Marine Biological Laboratory, Woods Hole, Mass.

One of the factors considered to be responsible for the different respiratory response to fertilization of the eggs of different animals is the stage of maturation at which fertilization takes place (for a discussion, see Rothschild, 1956; Monroy, in press). This evidently implies also a different metabolic condition of various eggs at the moment of fertilization. On the contrary, according to one of the present authors (Monroy, in press), all eggs are fertilized in a fundamentally similar physiological condition, independent of the stage of maturation attained. It has been suggested that in the course of the intra-ovarian maturation a metabolic block builds up progressively within the egg. When the threshold concentration of the inhibiting factor(s) is reached, maturation is arrested. The stage to which nuclear maturation has progressed would thus indicate the moment when such a threshold concentration has been attained.

Hence, at fertilization all eggs should be released from a metabolic inhibition. If this is correct, and the results to be presented here suggest that it may indeed be so, the next question is: does the release of the inhibition immdiately follow fertilization or does it require that maturation be completed first in the case of those eggs which are fertilized before the initiation or the completion of maturation? The present experiments provide also some information on this question.

One of the most impressive indications of the metabolic inhibition of the unfertilized sea urchin egg is its inability to carry out protein synthesis. In fact, although unfertilized sea urchin eggs take up labelled amino acids, these are not (or to a negligible extent) incorporated into proteins (Hultin, 1950, 1952; Hoberman, Metz and Graff, 1952; Nakano and Monroy, 1958; Monroy, 1960). Particularly striking have been the results obtained with C^{14}-glucose, which is taken up by the unfertilized egg and used for the synthesis of alanine, serine, aspartic and glutamic acid. However, no incorporation of these amino acids into proteins occurs prior to fertilization (Monroy and Vittorelli, 1962). Incorporation into proteins of amino acids either administered from without or already present in the egg begins immediately following fertilization. On the other hand, incorporation of amino acids in the proteins is quite active in the oocytes (see Monroy and Maggio, in press), which is in agreement with the notion of the high rate of oxygen consumption of the oocyte and of its decline during maturation (Lindahl and Holter, 1941).

---

[1] This investigation was carried out during the summer of 1963 and was sponsored by the Training Grant 5 T1GM 998-02 (Physiology of Gametes and Fertilization) of the National Institutes of Health.

[2] Present address: Istituto di Anatomia Comparata, Università di Palermo, Palermo, Italy.

[3] Present address: Biology Department, Brown University, Providence, R. I.

In the experiments presented in this paper the rate of incorporation of labelled amino acids and of C^{14} from glucose into the proteins has been investigated in the eggs of *Asterias forbesii* and *Spisula solidissima*. The ripe eggs of *Asterias* are shed with an intact germinal vesicle which ruptures as soon as the eggs reach the sea water. Maturation then progresses until the formation of the two polar bodies. The eggs can be inseminated at any moment during this cycle although the percentages of fertilization and cleavage are higher when the eggs are fertilized between the rupture of the germinal vesicle and the formation of the first polar body. Fertilization is accompanied by the formation of the fertilization membrane but the sperm nucleus remains "idle" beneath the egg cortex until the completion of maturation.

In the eggs of *Spisula*, on the contrary, the breakdown of the germinal vesicle, *i.e.*, the beginning of maturation, is induced by fertilization.

The eggs of *Asterias* and *Spisula* were selected for experiment because of the different conditions under which maturation is initiated; this should permit useful comparison with the previous results obtained with the sea urchin egg. A preliminary report of these results has been already published (Tolis and Monroy, 1963a, 1963b).

MATERIAL AND METHODS

Preparation of the egg

Ripe gametes were obtained from *Asterias* by an injection of a preparation of the spawning factor of Chaet (1964); spawning followed within 30–45 minutes. The eggs were suspended in sea water, centrifuged gently, re-suspended in sea water and divided into two equal batches. One batch was left unfertilized while the other was fertilized; both batches were allowed to stand in large fingerbowls at about 18° C., and measured aliquots were collected at the desired times for the exposure to the radioactive compounds. The time elapsed between spawning and fertilization usually did not exceed 10 minutes. One hundred per cent fertilization was the rule; normal cleavage occurred in 80–95% of the eggs. Under the conditions of our experiments the formation of the polar bodies occurred simultaneously in the unfertilized and fertilized eggs, the first being formed about 40 minutes after shedding and the second about 20 minutes later. Because of the large quantities of eggs obtained, the eggs of a single female were used for each experiment. Eggs were artificially activated by a 5-minute treatment with the Ca-Na hypertonic sea water as suggested by R. S. Lillie (1941), *i.e.*, 10 ml. of 2.5 *M* Ca-Na solution (95 ml. of 2.5 *M* NaCl + 5 ml. of 2.5 *M* $CaCl_2$) + 50 ml. sea water.

The gametes of *Spisula* were obtained and handled as suggested by Allen (1953). Under these conditions, 100% fertilization and cleavage were obtained. The eggs were cultured in a thin layer in large fingerbowls. Breakdown of the germinal vesicle took place between 9 and 12 minutes after fertilization; the first polar body was formed about 30 minutes after fertilization and the second 20 minutes later.

Pulse labelling

The eggs were exposed to the radioactive compounds at certain stages during maturation and fertilization. In the experiments with labelled amino acids, strong labelling was obtained with 5-minute pulses, whereas in the case of glucose an exposure of 30 minutes was necessary. Hence in the latter case the definition of the stages was less precise. In the case of *Asterias* the first pulse was given as soon as in the batch of fertilized eggs the fertilization membrane appeared; then at the time of the formation of the first and second polar bodies and finally twice more during cleavage (see figures). In the experiments with *Spisula* eggs the germinal vesicle breakdown stage was also included.

The following procedure was followed throughout the experiments both with *Asterias* and *Spisula*. A measured aliquot of egg suspension was pipetted into a 10-ml. Erlenmeyer flask; the solution of the labelled compound was then added to a final concentration of: C^{14}-D-glucose (U):0.5 μC./ml. (specific activity 3.83 μC./ /μM); S^{35}-L-methionine:1 μC./ml. (specific activity 13.4 μC./μM); L-valine-1-C^{14}:1 μC./ml. (specific activity 5.9 μC./μM). The labelled compounds were added simultaneously to the flasks of the fertilized and unfertilized eggs. In the glucose experiments at the end of the treatment, the eggs were rapidly centrifuged (a hand centrifuge has been used for such washings), washed once with ice-cold sea water containing 1% C^{12}-D-glucose and three times with sea water. In the amino acid experiments, the uptake was stopped by the addition of an excess of cold amino acid in sea water to bring the final concentration to 1 mg./ml.; after centrifugation, the eggs received three more washings with sea water. Further processing was the same in all experiments. The whole washing procedure did not take more than 10 minutes. The packed eggs were taken up in 1 ml. of ice-cold distilled water and transferred to a homogenizer in which they were rapidly homogenized. From the homogenates, two 0.1-ml. samples were collected for the estimation of total nitrogen and three 0.1-ml. samples were rapidly dried on filter paper discs. One of the discs was used for the estimation of total uptake; the other two were extracted with TCA and alcohol-ether, as suggested by Mans and Novelli (1960). Counting was done in a Packard scintillation counter at 50% efficiency. Total nitrogen was estimated by Nesslerization after combustion.

RESULTS

1. Asterias forbesii

(a) *Experiments with C^{14} glucose.* Preliminary experiments carried out by the senior author on the egg of the Mediterranean *Asterias glacialis* showed that both unfertilized and fertilized eggs were able to utilize the carbon from glucose for the synthesis of glutamic and aspartic acid and alanine. The curves of Figure 1 show that in the unfertilized eggs the rate of total uptake underwent a sharp decline until after the formation of the second polar body and then remained almost constant. In the fertilized eggs, during the first half hour after fertilization (approximately until the formation of the first polar body) the rate of uptake was lower than in the unfertilized eggs, but then it did not undergo the rapid drop observed in the unfertilized eggs. Indeed, it remained almost constant until completion of maturation and then began slowly to increase. The significance of the lower rate of uptake of the fertilized eggs during the first half hour following fertilization is at present obscure. A small increase in the rate of incorporation into the proteins during maturation was sometimes observed both in the unfertilized and fertilized eggs (as in the experiment used for Figure 1). In other cases (as in the experiment used in Table 1) no such increase occurred until after the formation of the second polar body. After the completion of maturation, the rate of incorporation rose in the fertilized eggs, whereas it remained either constant or declined in the unfertilized eggs. It must be noted, however, that the values of

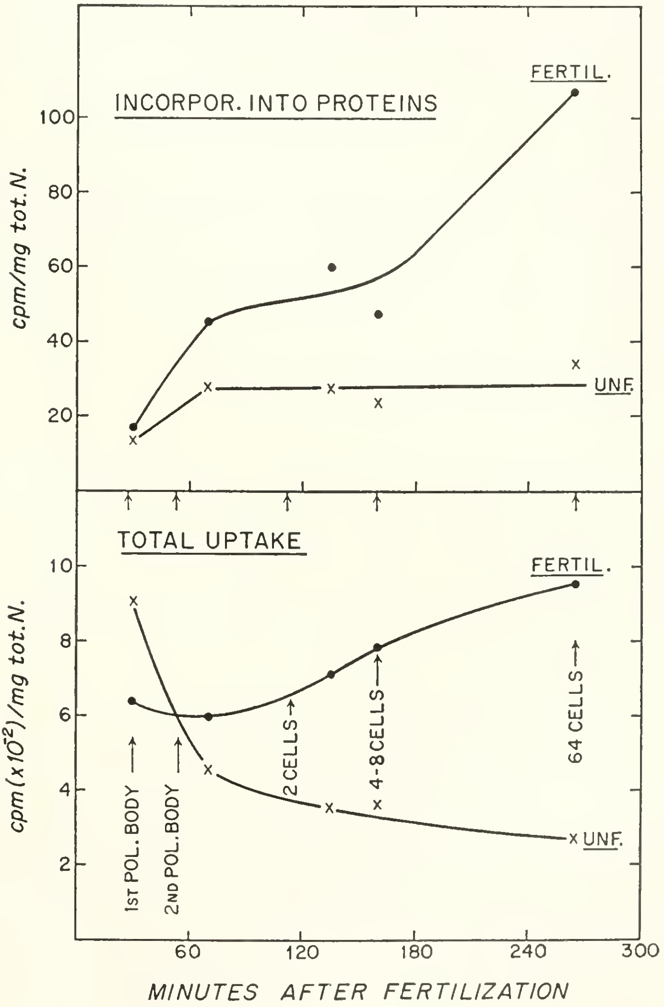


Figure 1. *Asterias forbesii.* Rate of total uptake and incorporation into proteins of C^{14} from glucose. The position of the points of the curves is at the end of the 30-minute pulse. For the interpretation of the incorporation curve see the text.

incorporation were always fairly low and therefore the only safe statement that can be made concerns the increase that occurs in the fertilized eggs after maturation has been completed. More extensive experiments are required to check the actual shape of the incorporation curve during cleavage.

Spontaneous activation of the unfertilized eggs was observed in some experiments. Activation was indicated by the elevation of a fertilization membrane and centralization and swelling of the nuclei. Activation was always accompanied by a sudden and transient increase in the rate of total uptake. The rate of incorporation into the proteins, on the contrary, did not change appreciably (Table I). Experiments of parthenogenetic activation were carried out using the hypertony method of R. S. Lillie (see Methods). Artificial activation, accomplished soon after the eggs had been shed (*i.e.*, following the same schedule as in the fertilization experiments), was followed by an immediate increase in the rate of uptake.

TABLE I

Rate of uptake and of incorporation into the proteins of C^{14} from glucose in spontaneously (column 1) and artificially (column 4) activated Asterias eggs. Spontaneous activation took place independently of any treatment, in the course of an experiment at the indicated time; artificial activation was carried out 10 minutes after shedding. In both experiments time is indicated from fertilization or artificial activation.

The data in column 1 should be compared with those in column 2 (fertilized eggs of the same batch), and those in column 4 with those in column 3 (unfertilized eggs of the same batch). Radioactivities are expressed as C.P.M./mg. total nitrogen.

Time	1 Unfertilized		2 Fertilized		Time	3 Unfertilized		4 Activated	
	Uptake	Incorp.	Uptake	Incorp.		Uptake	Incorp.	Uptake	Incorp.
30	1010	15.1	935	19.3	30	810	31.2	1565	42.6
60	650	16.4	814	15.5	150	585	9.8		
140	496	lost	930	40.5					
	Activ.—				210	481	3.3	1605	64.2
190	920	15.0	1214	73.5					
300	733	7.2	1260	72.5					

Thus, whereas in the fertilized eggs there is a transient period during which the permeability appears to be lowered, in the activated ones it always undergoes an immediate increase. This seems to suggest a different type of surface change in the activated with respect to the fertilized egg. On the contrary, the increase in the rate of incorporation into the proteins was smaller than in the fertilized eggs (Table I). Also in the sea urchin eggs the amino acid incorporation into proteins in butyric acid-activated eggs was lower than in the fertilized ones (Nakano, Giudice and Monroy, 1958).

(b) *Experiments with S^{35}-L-methionine and L-valine-1-C^{14}.* The experiments with valine (Fig. 2) duplicate quite well the results obtained with glucose. Indeed, in these experiments a decline of the rate of uptake was observed during maturation, which was less pronounced in the fertilized than in the unfertilized eggs. Again the first pulse (which, as mentioned, in these experiments was of 5

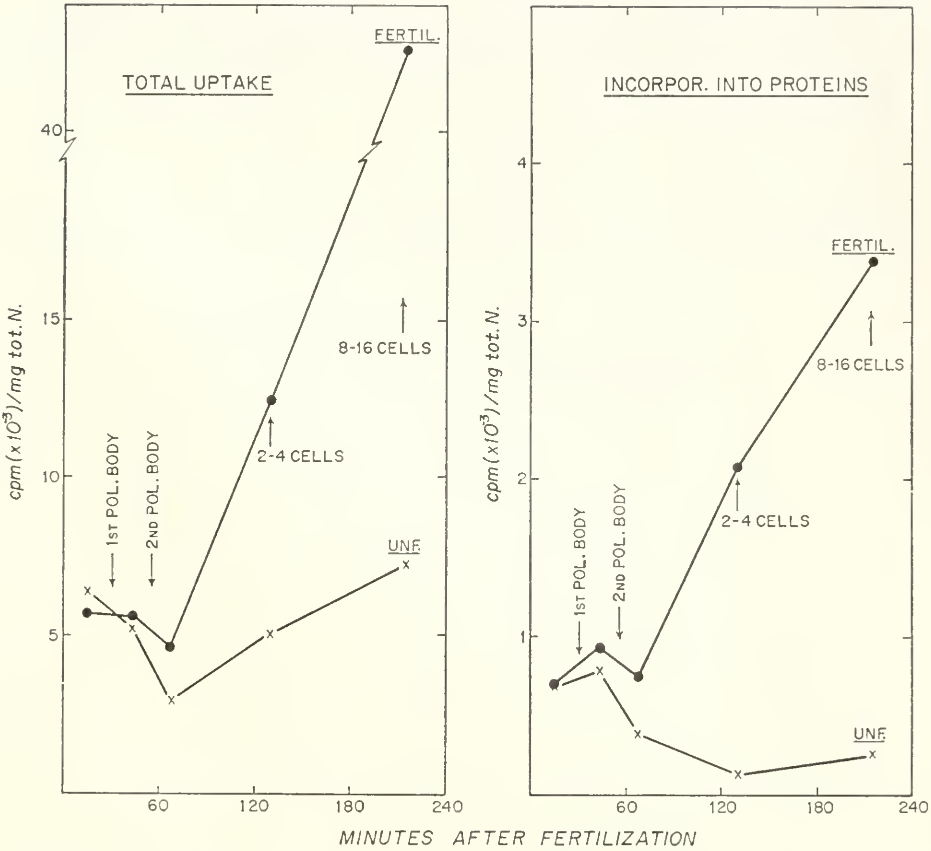


FIGURE 2. *Asterias forbesii.* Rate of total uptake and incorporation into proteins of L-valine-1-C^{14} in unfertilized and fertilized eggs. At the time of the fourth pulse, 40% of the unfertilized eggs had undergone activation and at the time of the last pulse nearly 90% had membranes and centralized and swollen nuclei.

minutes) showed a slightly lower rate of total uptake in the fertilized than in the unfertilized eggs. After completion of maturation the increase in the rate of total uptake in the fertilized eggs was far greater than in the glucose experiments. In the experiment used for the preparation of Figure 2, the unfertilized eggs underwent spontaneous activation soon after the end of maturation and also in this case a slight increase of the rate of uptake took place. In another experiment in which this did not take place, no such increase was observed.

When methionine was tested (Fig. 3) the rate of uptake in the unfertilized eggs increased slightly but linearly during the four hours of the experiment; no change was observed in conjunction with the completion of maturation. Furthermore, until the formation of the second polar body, the values of the rate of uptake were practically identical in the unfertilized and in the fertilized eggs. Afterwards a dramatic increase was observed in the fertilized egg. Both in the unfertilized and fertilized eggs the rate of incorporation of valine and methionine into proteins increased slightly during maturation. Once maturation was completed, the rate of incorporation declined in the unfertilized eggs while it increased considerably in the fertilized ones.

2. *Spisula*

Only experiments with valine were performed on these eggs. A typical experiment is summarized in Figure 4. In the unfertilized egg the rate of uptake re-

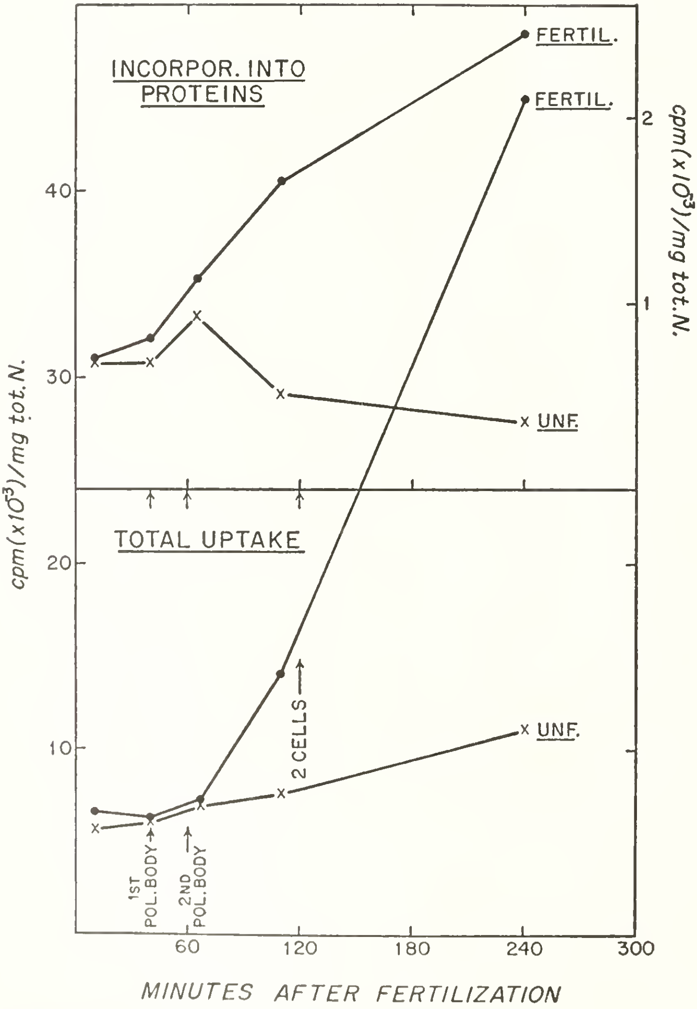


FIGURE 3. *Asterias forbesii.* Rate of total uptake and incorporation into proteins of S^{35}-L-methionine. The figures on the left ordinate refer to the total uptake and those on the right one to the incorporation into proteins.

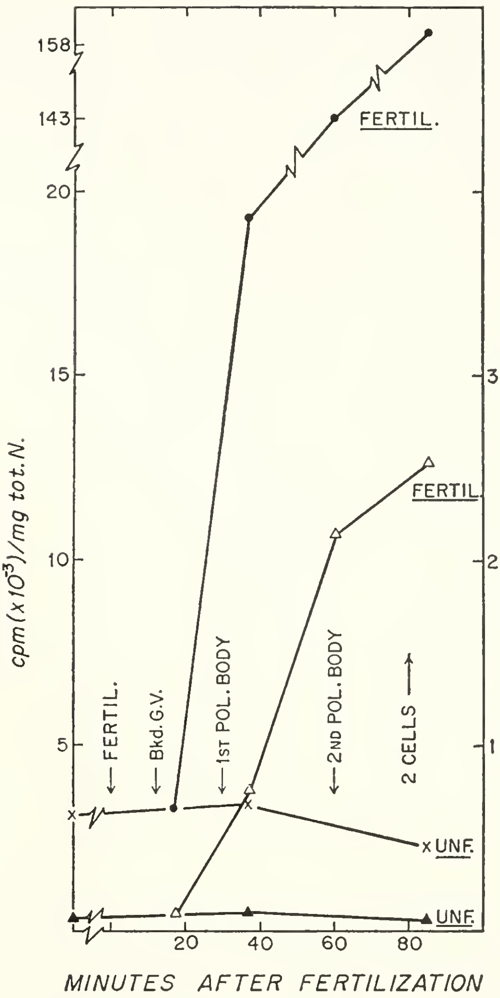


FIGURE 4. *Spisula solidissima.* Rate of total uptake (x——x, unfertilized eggs; ●——●, fertilized eggs) and incorporation into proteins (▲——▲, unfertilized eggs; △——△, fertilized eggs) in eggs pulsed with L-valine-1-C^{14}. Values on the left ordinate refer to total uptake and on the right ordinate to incorporation into proteins.

mained almost constant for about 60 minutes and then declined slightly. The rate of incorporation into proteins was low and remained constant (Fig. 4). When a 5-minute pulse was given to fertilized eggs in which the germinal vesicle had just broken down (the breakdown of the germinal vesicle usually started about 10 minutes after insemination and was complete in about 5 minutes) the rate of total uptake and of incorporation was the same as one would expect in the unfertilized eggs (Fig. 4). Indeed, the experimental points fall exactly on the expected line of the unfertilized eggs. However, a pulse applied about 20 minutes later (*i.e.*, 5 minutes after the formation of the first polar body) showed a great increase in the rate both of uptake and of incorporation (Fig. 4). Indeed at the completion of maturation the rate of uptake was more than 40 times greater than that in the unfertilized egg, and the rate of incorporation into proteins had increased 20 times. The increase continued during the first cleavage but was not as rapid as during maturation.

Discussion

The results of the experiments described in this paper support the hypothesis that at the moment of fertilization all the eggs, irrespective of the stage of maturation reached, are in a metabolically inhibited condition. In fact both the eggs of *Asterias* and of *Spisula*, in spite of their being shed as primary oocytes, have proved to have very little, if any, ability to carry out incorporation of amino acids into proteins. Hence they are in an analogous condition to the fully mature sea urchin egg before fertilization. A comparison of these results with the respiratory metabolism may prove especially illuminating. Nothing is known about the respiratory changes of the *Spisula* egg following fertilization. As for the *Asterias* egg, according to Borei (1948), at the onset of maturation the rate of oxygen consumption begins to increase; it reaches a peak at the time of the first meiosis and then starts to decline. Hence in this respect the starfish egg would appear to behave differently from the sea urchin (Lindahl and Holter, 1941) and fish (Nakano, 1953) oocytes, in which the rate of oxygen consumption rapidly declines at the onset of maturation. Following fertilization the respiratory rate increases slowly. However, if the slower rate of cleavage of the starfish as compared to the sea urchin egg is taken into consideration, then the two respiratory curves turn out to be well superimposable (Borei and Lybing, 1949). In our experiments, a decline in the rate of uptake of glucose and valine during maturation has been observed both in the unfertilized and fertilized eggs whereas in the methionine experiments it remained practically constant. Thus, the curves for glucose and valine are similar to the respiratory curves of the maturing sea urchin and fish oocytes. On the other hand, the curve of incorporation into the proteins is similar to the respiratory curve described by Borei (1948) in starfish. It must be emphasized that work with starfish oocytes and eggs has been hampered by the difficulty of obtaining homogeneous batches of eggs. Now that this difficulty has been largely overcome (Chaet, 1964), a re-examination of the respiration of the starfish oocyte during maturation and fertilization becomes imperative. However, another possibility to explain the decrease in the uptake of glucose and valine during maturation is that it may simply depend on the decreased permeability of the egg upon standing in sea water; such a decrease would apparently be somewhat lessened by fertilization. This possibility is born out of the observation of Dalcq (1924) that the resistance of the starfish oocyte to cytolysis by dilute sea water increases during maturation. The acceleration of incorporation observed during maturation both in the unfertilized and in the fertilized eggs is worth mentioning. Whether or not this acceleration is connected with the maturation divisions (formation of the spindle?) is at present obscure.

The present experiments have also provided some information pertaining to the second question raised in the introduction, namely whether the inhibition of the unfertilized egg is released immediately following fertilization, as in the case of the sea urchin egg, or if in the eggs which are fertilized before the initiation or in the course of maturation, this must be completed first. In this respect, the eggs of *Asterias* and of *Spisula* have behaved differently. In fact, in the former the release occurs only after the completion of maturation, in the latter soon after the breakdown of the germinal vesicle and well before the formation of the first polar body. Our data, although limited to the eggs of only two animal species, show that the inhibition is not released *immediately* following fertilization. Indeed, maturation must progress to a certain stage, different in the eggs of the different animal groups, for such a release to occur.

The authors wish to express their gratitude to Dr. Ch. B. Metz, for his continuous help and interest during the operation of the Training Program. They are also indebted to Dr. A. B. Chaet for his assistance in the use of his spawning factor of *Asterias;* to Dr. L. Rebhun for his advice in the use of the eggs of *Spisula* and to Dr. J. Morrill for reading the manuscript.

SUMMARY

1. In the eggs of *Asterias forbesii* the rate of total uptake of C^{14}-glucose and of L-valine-1-C^{14} declines during maturation both in unfertilized and fertilized eggs. The rate of uptake of S^{35}-L-methionine remains constant throughout maturation. After completion of maturation the rate of uptake is greatly increased in the fertilized eggs, whereas in the unfertilized ones it either continues to decline or remains constant.

2. The rate of incorporation of C^{14} from glucose and of C^{14}-valine and S^{35}-methionine into proteins undergoes a slight acceleration during maturation both in unfertilized and fertilized eggs. After the formation of the second polar body, the rate of incorporation declines in the unfertilized eggs while continuing to increase in the fertilized ones.

3. Activation, either spontaneous or artificial, brings about a rapid increase of the rate of uptake of the precursors used.

4. In the eggs of *Spisula* the rate of uptake of L-valine-1-C^{14} and of its incorporation into proteins is very low in the unfertilized eggs. A tremendous increase both in the rate of uptake and of incorporation into proteins takes place a few minutes after the fertilization-induced breakdown of the germinal vesicle.

5. The significance of these observations is discussed in relation to the problem of the release of the metabolic inhibition of the unfertilized egg upon fertilization, and in comparison with the results obtained in the sea urchin egg.

LITERATURE CITED

ALLEN, R. D., 1953. Fertilization and artificial activation in the egg of the surf-clam, *Spisula solidissima*. *Biol. Bull.*, **105**: 213–239.

BOREI, H., 1948. Respiration of oocytes, unfertilized eggs and fertilized eggs from *Psammechinus* and *Asterias*. *Biol. Bull.*, **95**: 124–150.

BOREI, H., AND S. LYBING, 1949. Oxygen uptake of *Asterias* embryos before hatching. *Nature*, **163**: 451–452.

CHAET, A. B., 1964. A mechanism for obtaining mature gametes from starfish. *Biol. Bull.*, **126**: 8–13.

DALCQ, A., 1924. Recherches expérimentales et cytologiques sur la maturation et l'activation de l'oeuf d'*Asterias glacialis*. *Arch. Biol.*, **35**: 507–674.

HOBERMAN, H. D., C. B. METZ AND J. GRAFF, 1952. Uptake of deuterium into proteins of fertilized and unfertilized *Arbacia* eggs suspended in heavy water. *J. Gen. Physiol.*, **35**: 639–643.

HULTIN, T., 1950. The protein metabolism of sea urchin eggs during early development studied by means of N^{15}-labelled ammonia. *Exp. Cell Res.*, **1**: 599–602.

HULTIN, T., 1952. Incorporation of ^{15}N-labeled glycine and alanine into the proteins of developing sea urchin eggs. *Exp. Cell Res.*, **3**: 494–496.

LILLIE, R. S., 1941. Further experiments on artificial parthenogenesis in starfish eggs, with a review. *Physiol. Zoöl.*, **14**: 239–268.

LINDAHL, P. E., AND H. HOLTER, 1941. Über die Atmung der Ovozyten erster Ordnung von *Paracentrotus lividus* und ihre Veränderung während der Reifung. *C. R. Tr. Lab. Carlsberg, Sér. Chim.*, **24**: 49–57.

MANS, R. J., AND G. D. NOVELLI, 1960. A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. *Bioch. Bioph. Res. Comm.*, **3**: 540–543.

MONROY, A., 1960. Incorporation of S^{35}-methionine in the microsomes and soluble proteins during the early development of the sea urchin egg. *Experientia*, **16**: 114–117.

MONROY, A. Biochemical aspects of fertilization. *In:* Biochemical Aspects of Development, R. Weber, Editor, Academic Press, New York. (In press.)

MONROY, A., AND M. L. VITTORELLI, 1962. Utilization of ^{14}C-glucose for amino acids and protein synthesis by the sea urchin embryo. *J. Cell. Comp. Physiol.*, **60**: 285–288.

MONROY, A., AND R. MAGGIO. Biochemical studies on the early development of the sea urchin. *Adv. in Morphogenesis*, **3** (in press).

NAKANO, E., 1953. Respiration during maturation and at fertilization of fish eggs. *Embryologia*, **2**: 21–31.

NAKANO, E., G. GIUDICE AND A. MONROY, 1958. On the incorporation of S^{35}-methionine in artificially activated sea urchin eggs. *Experientia*, **14**: 11–13.

NAKANO, E., AND A. MONROY, 1958. Incorporation of S^{35}-methionine in the cell fractions of sea urchin eggs and embryos. *Exp. Cell Res.*, **14**: 236-244.

ROTHSCHILD, LORD, 1956. Fertilization. Methuen & Co., London.

TOLIS, H., AND A. MONROY, 1963a. Uptake of C^{14}-glucose by unfertilized and fertilized eggs of *Asterias forbesii*. *Biol. Bull.*, **125**: 368–369.

TOLIS, H., AND A. MONROY, 1963b. Uptake and incorporation of radioactive amino acids in unfertilized and fertilized eggs of *Asterias forbesii* and *Spisula solidissima*. *Biol. Bull.*, **125**: 369.

STUDIES ON THE TREMATODE GENUS RENICOLA: OBSERVATIONS ON THE LIFE-HISTORY, SPECIFICITY, AND SYSTEMATIC POSITION [1]

HORACE W. STUNKARD

The American Museum of Natural History, Central Park West at 79th Street, New York 24, New York

The genus *Renicola* was erected by Cohn (1904) to contain *Monostomum pingue* (Mehlis in Creplin, 1846), when he discovered that the species has a distinct acetabulum and could no longer be regarded as a monostome. He studied the original material of Mehlis, from the kidneys of the crested grebe, *Podiceps cristatus*, in both whole-mount preparations and serial sections. He noted that the worms, although hermaphroditic, live in pairs in expansions of the renal tubules, that there is no copulatory organ, and that the acetabulum is so obscured by eggs in the uterus that it is quite invisible in whole-mount preparations. In this paper he advanced the idea that adhesive organs of trematodes which live in closed cavities do not function actively and accordingly either fail to develop or undergo atrophy and become reduced in size.

The second and third species of the genus, *Renicola secunda* and *R. tertia*, were described by Skrjabin (1924) from the white pelican, *Pelecanus onocrotalus*, and from the tern, *Sterna fluviatilis*, respectively, both taken in Russian Turkestan. The descriptions are brief, listing sizes of body and of eggs, but the figures give other pertinent information. The three species of *Renicola* were characterized by shape of body, including the degree of posterior extension, the relative length of the digestive ceca, and the location and extent of the vitellaria. However, Skrjabin stated that because of meager material, specific determination was uncertain. Yamaguti (1958) listed *Renicola zarudni* Skrjabin, 1927, from a pelican taken by the 28th Helminthological Expedition of the U.S.S.R. to Turkestan. However, there is no reference to *R. zarudni* in Skrjabin, Trematodes of Animals and Man, vol. 1, pp. 261–277 (1947), which deals with the genus *Renicola*. Odening (1962b) listed *R. zarudni* as a *nomen nudum* and the species as identical with *R. secunda* Skrjabin, 1924.

The next species of the genus was described by Witenberg (1929) from *Puffinus kuhli*, taken by the Sinai Expedition of the Hebräischen Universitaet, Jerusalem, to the Suez. Witenberg noted that the descriptions of the previously named species were so incomplete that determination of new material should be made with caution. To identify the specimens from *P. kuhli*, they were designated provisionally as a new species, *Renicola glandoloba*, although Witenberg admitted the possible identity of the previously described species with the new species described by him.

---

[1] This investigation was supported by Grant NSF-G23561.

Timon David (1933) described *Renicola lari* from the renal tubules of the herring gull, *Larus argentatus*, taken near Marseille, France. The account by Timon David was supplemented by Dollfus (1946) who studied many preparations provided by Dr. Timon-David and gave a more detailed and precise figure of the species. Since that time, the number of described species has mounted, but specific distinctions have become more and more obscure. In chronological sequence they are:[2]

6. *R. quintus* Sokolowa-Andronowa, 1937, from *Pseuduria carbo*, in Russia.
7. *R. paraquintus* Rayevsky, 1937, from *Larus ridibundus*, in Vladivostok, Russia.
8. *R. keimahuri* Yamaguti, 1939, from *Uria carbo*, in Japan.
9. *R. umigarasu* Yamaguti, 1939, from *Uria aalge inornata*, in Japan.
10. *R. pandioni* Sudarikov, in Skrjabin, 1947, from *Pandion haliaetus*, in Russia.
11. *R. undecimus* Sudarikov, in Skrjabin, 1947, from *Pandion haliaetus*, in Russia.
12. *R. vladika* Oschmarin, 1950, from *Phalacrocorax pelagicus*, in Kamtschatka, Russia.
13. *R. magnicaudatus* Bykhovskaia-Pavlovskaia, 1950, from *Hirundo rustica*, in Siberia.
14. *R. mediovitellatus* Bykhovskaia-Pavlovskaia, 1950, from *Nyroca ferina*, *Anas strepera*, and *Spatula clypeata*, in Siberia.
15. *R. brantae* McIntosh and Farr, 1952, from *Branta canadensis*, in North Carolina.
16. *R. murmanicus* Belopolskaia, 1952, from *Larus canus*, in Russia.
17. *R. somateriae* Belopolskaia, 1952, from *Somateria spectabilis*, in Russia.
18. *R. bretensis* Timon-David, 1953, from *Pica, pica*, in Southern France.
19. *R. thapari* Caballero y C., 1953, from *Pelecanus occidentalis californicus* in the Canal Zone, Central America.
20. *R. nanus* Bykhovskaia-Pavlovskaia, 1954, from *Tringa totanus* and *Vanellus vanellus*, in Siberia, Russia.
21. *R. cruzi* Wright, 1954, from *Sterna maxima* and *Sterna hirudinacea*, in Brazil.
22. *R. pelecani* Wright, 1954, from *Pelecanus onocrotalus* and *Pelecanus philippensis*, in India and Ceylon.
23. *R. sloanei* Wright, 1954 from the common guillemot, *Uria aalge*, taken in Sussex, England and the first wild bird in Great Britain found infected with renicolid trematodes; the ringed penguin, *Pygoscelis antarctica* taken in South Georgia; and the macaroni penguin, *Eudyptes chrysolophus*, which had been received from the Edinburgh Zoo. The specimen of *U. aalge* had been in the Zoo only 14 days; the ringed penguin, 18 months; and no information is supplied concerning the time the macaroni penguin had been in the Zoo. Campbell and Sloane (1943) had reported this species, but without name, from a number of king

---

[2] Footnote from Odening, 1962b, p. 58, "Das grammatische Geschlecht des Gattungsnamens wird von der Mehrzahl der Autoren als weiblich angesehen, von Yamaguti (1958) jedoch aus philologisch wahrscheinlich gerechtfertigten Gründen als männlich. Hier wird der Einheitlichkeit halber die weibliche Auslegung beibehalten."

penguins, *Aptenodytes longirostris;* a black-footed penguin, *Spheniscus demersus;* and a Gentoo penguin, *Pygoscelis papua*, all of which had died in Edinburgh. These records suggest that the infection may have originated in the Edinburgh Zoo. Wright (1956) reported that the specific description of *R. sloanei* was based on specimens from the ringed penguin.

24. *R. mirandariberoi* Teixera de Freitas, 1955, from *Sula leucogaster,* in Brazil.

25. *R. goliath* Wright, 1957, from *Ardea goliath*, in the Sudan, Africa.

26. *R. mollissimus* Kulachkova, 1957, from *Somateria mollissima*, in Russia.

27. *R. monorchis* Dollfus and Capron, 1958, from *Necrosyrtes monachus*, in Senegal, Africa.

28. *R. philippinensis* Stunkard, Nigrelli and Gandal, 1958, from *Hydropasianus churungus*, taken in the Philippine Islands, died in the Bronx Zoo, New York.

29. *R. sudaricovi* Leonov, 1958, from herons, "Reihervögeln," in Russia.

30. *R. pollaris* Kontrimavitschus and Bakhmeteva, 1960, from *Gavia stellata*, Lena River, Russia.

31. *R. vietnamensis* Odening, 1962, from *Nycticorax nycticorax*, taken in Vietnam, died in the Berlin Zoo, 68 days after arrival.

32. *R. fischeri* Odening, 1962, from *Ardea bacchus*, taken in Vietnam, died in the Berlin Zoo, 141 days after arrival.

33. *R. dollfusi* Odening, 1962, (=*Renicola* sp. Dollfus, 1946).

34. *R. pseudosloanei* Odening, 1962 (=*R. sloanei* Wright, 1954 *ex parte*, from *Uria aalge; Renicola* sp. Wright, 1956, from *Uria aalge; Renicola* sp. Wright, 1956, from *Gavia arctica*.

35. *R. wrighti* Odening, 1962 (=*Renicola* sp. Wright, 1956, from *Fratercula arctica; Renicola* sp. Wright, 1956 from *Puffinus puffinus*).

36. *R. indicolus* Odening, 1962 (=*Renicola* sp. Wright, 1954 from *Pelecanus onocrotalus; Renicola* sp. Odening, 1962, from *Pelecanus philippensis*, died soon after arrival at the Berlin Zoo.

37. *R. brevivitellus* Leonov and Belogurov, 1963, from *Sterna camtschatica* and *Sterna hirundo*, Aleutian Islands.

38. *R. hydranassae* Lumsden and Zische, 1963, from a Louisiana heron, *Hydranassa tricolor*. These authors also described a second species, which differed from descriptions of *R. glandoloba* only in slightly smaller eggs.

Gubanov (1954) reported a renicolid species from *Fulmaris glacialis* taken at the Ochtotskisches Meer in Russia but there was no description and the name, *R. fulmari*, remains a *nomen nudum*.

In addition to the named species, many others have been reported and described but not named. Sokolova-Andronova (1937) reported renicolid trematodes from the kidneys of *Phaleris psittacula* and *Melanitta fusca* from the Far-East, Russia, but there were no figures or descriptions. Dollfus (1946) prepared a catalogue of the trematodes from the renal organs of birds, revised the family Renicolidae Dollfus, 1939 and devised a key for the identification of the species in *Renicola*. In addition, he described and figured, but did not name, a species from *Plotus* (=*Mergulus*) *alle*, killed at Wimereux, Pas-de-Calais, France. The material consisted of five specimens, four of which were immature, found in the digestive ceca of the bird. He stated that the location is probably accidental, the worms having migrated to the cloaca and subsequently to the ceca after the death of the host. Dollfus noted that in living worms, the caudal prolongation of the body disappears when the posterior region is retracted, and that the relative position of the acetabulum shifts with the extension and retraction of the anterior and posterior portions of the body. He also noted and figured variation in the shape and size of eggs from a single specimen. In the same issue of the Annales de Parasitologie, Callot (1946) described an unnamed species of *Renicola* from *Sterna cantiaca* "provenant des côtes de la Manche," probably not far from Wimereux. Wright (1954a), in addition to the description of *R. cruzi*, described three unnamed species from South American birds: one from *Larus dominicanus*, one from *Poecilonetta bahamensis*, and the other from *Sula leucogaster*.

Wright (1954b) reported on renicolid trematodes from birds that had died in the London Zoo and from others obtained from the Veterinary Laboratories Poultry Advice Service Department at Weybridge. *Renicola pelecani* was described as a new species from the spot-billed pelican, *Pelicanus philippensis* from Ceylon and white pelicans, *Pelecanus onocrotalus*, from Calcutta, India. The spot-billed pelican died one day after arriving at the Zoo and two white pelicans 21 and 28 days, respectively, after their arrival, which strongly indicated that the infection had been acquired in India. A third white pelican, which had been in the Zoo for seven months, yielded two worms, one of which was badly damaged; the genital organs and ventral sucker of the other were completely obscured by the dense mass of eggs in the uterine sac. However, the author noted a resemblance between them and *R. secundus* Skrjabin, 1924. After the description of *R. sloanei*, Wright predicated that it may be identical with the unnamed species described by Dollfus (1946) from *Mergulus alle*. In addition to *R. pelecani* and *R. sloanei*, Wright described other unnamed members of the genus *Renicola*, one species from the black-headed ibis, *Threskiornis melanocephala*; two species from the white pelican, *P. onocrotalus*; and another species from the cattle egret, *Bulbulcus coromandus*, listed, but not described, by Hammerton in the annual report of the Prosector to the Zoological Society of London for 1933. Certain of these species, as noted earlier, were named by Odening (1962b), but mere naming, while it facilitates identification of particular specimens, adds nothing to knowledge of the genus *Renicola*.

Wright (1956) presented further data on renicolid trematodes of British birds and an account of efforts to elucidate their life-cycles. Much of the material was obtained from oiled and dead sea-birds, washed up on the coast during the winter seasons. In addition to specimens described in his earlier reports, Wright described worms taken from *Uria aalge*, *Colymbus arcticus*, *Fratercula arctica* and *Puffinus puffinus*. The worms from *C. arcticus* and *F. arctica* were similar to *R. sloanei*. Those found in *P. puffinus* were allocated to two species on differences in the extent of the vitellaria, although in other characters the two forms intergrade.

Other unnamed species have been reported by Russian investigators. One was described by Ginetzinskaia (1952) from *Fulica atra* taken in the Volga delta. Three unnamed species were recorded by Bykhovskaia-Pavlovskaia (1962); one from *Plegadis falcinellus* taken in the Volga delta; one from *Calidris martima* taken at the Barents See; and the third from the Far-East region of Russia.

Previously, Nezlobinsky (1926) had described a new genus and species, *Stamparia macedoniensis*, from the kidneys of *Pelecanus onocrotalus* taken in Macedonia. Caballero y C. (1953) declared that *Stamparia* is a synonym of *Renicola* and that *R. lari* Timon-David, 1933 is identical with *R. glandoloba* Witenberg, 1929. Subsequent authors, Wright (1956), Yamaguti (1958) and Odening (1962b), have concurred in the suppression of *Stamparia* as a synonym of *Renicola*.

Odening (1962a) described another unnamed species, *Renicola* sp., from *Pelecanus philippensis*, recently imported from India and dead in the Berlin Zoo. Odening (1962b) described *Renicola vietnamensis* n. sp., based on two worms from *Nycticorax nycticorax*, and *Renicola fischeri* n. sp., from *Ardeola bacchus*. He listed thirty species in the genus *Renicola*, analyzed the descriptions of several unnamed species, and provisionally allocated certain of them to new species, as listed earlier in the present paper. He arranged the species in groups and subgroups based on the length of the intestinal ceca; the ratio of sizes of the oral sucker, pharynx and acetabulum; the size of eggs; and the taxonomy of hosts. The genus was divided into three subgenera: *Renicola*, *Anatirenicola*, and *Wrightrenicola* on the location and extent of the vitellaria. Also, he proposed a new genus, *Neorenicola*, for *R. monorchis* Dollfus and Capron, 1958, and a new genus, *Pseudorenicola*, for *R. nana* Bykhovskaia-Pavlovskaia, 1954. Concerning the first of these species, Dollfus and Capron (1958) stated (p. 237), "Cette espèce appartient évidemment à la famille Renicolidae R.-Ph. Dollfus 1939 et au genre *Renicola* L. Cohn 1903, malgré son testicule unique, résultat de la coalescence de deux testicules comme le montrent la présence de deux spermiductes et sa forme bicorne." The genus *Pseudorenicola* was characterized by the postacetabular location of the ovary and the extent of the ceca into the hindbody, but these characters are not definitely associated since the ceca extend into the hindbody in *R. vietnamensis*, *R. fischeri*, and *R. hydranassae*, and the ovaries of these species are preacetabular.

The taxonomy of the renicolid trematodes is uncertain and somewhat confused. Odening (1962b) recounted the characters and proposed a revision of the Renicolidae. The family had been erected by Dollfus (1939) when he dismembered the heterogeneous family Troglotrematidae Odhner, 1914, and originally it contained only the genus *Renicola*. In his (1946) revision, Dollfus accepted the genus *Stamparia* Nezlobinsky, 1926 as a second genus in the family. This paper included a key to the nine species then included in the genus *Renicola* but the questionable status of the species is manifest by the statement (p. 55), "Chacune de ces espèces, sauf *lari*, n'a été vue qu'une seule fois, quelques-unes seulement en un ou deux exemplaires, aussi n'ont-elles pas pu être toutes suffisamment bien caractérisées, décrites et figurées. De nombreux individus sont presque toujours nécessaires pour une description détailée, et les descriptions d'après un très petit nombre d'individus, en plus ou moins mauvais état, ne pourront peut-être pas suffire pour que l'espèces soit reconnue avec certitude si elle vient à être retrouvée. L'anatomie d'une seule espèce a été décrite en détail, celle de *lari*, par J. Timon-David. Entre *lari* et *pinguis*, les différences sont extrêment faibles, *glandoloba* est bien peu différent et l'on ne connaît l'étendue des variations individuelles pour aucune des espèces." Since the statement by Dollfus (1946), the number of named species has increased from 9 to 38, and the situation reported by Dollfus has been aggravated, rather than clarified and simplified.

Wright (1956) deplored the incomplete descriptions of many species, which rendered recognition virtually impossible. He noted that Bykhovskaia-Pavlovskaia (1950) had recorded *R. lari* from *Hydrohelidon nigra, Sterna hirundo, Larus taimyrensis* and *Larus cachinans* taken in western Siberia; that McIntosh and Farr (1952) had suggested the identity of *R. brantae* McIntosh and Farr, 1952 with *R. mediovitellata* Bykhovskaia-Pavlovskaia, 1950; and that the status of *R. magnicaudata* Bykhovskaia-Pavlovskaia, 1950 and *R. nana* Bykhovskaia-Pavlovskaia, 1954 is doubtful and difficult to determine. He stated (p. 3), "During the course of the present work it has been found that variation between individual specimens from any batch of worms is so great that it must be accepted either that multiple infections with more than one species are common or that most previous workers have not taken into account the degree of variation within species. A study of the records of *Renicola* spp. together with their hosts shows that host-specificity in the sense of a group relationship does not apply in this genus. Apart from the Magpie, host of *R. bretensis*, the largely herbivorous anseriforms harboring *R. mediovitellata* and *R. brantae* and the two charadriiform hosts of *R. nana* all the other host species are almost exclusively fish-eaters. The Magpie is quite omnivorous and may easily eat fish when scavenging along the tide line, the Redshank and Lapwing both feed at least occasionally on the shore line and may take fish found washed up and the anseriforms are known to eat a considerable amount of animal food, particularly during the breeding season, but exact records of the nature of this food are few. The only feature in common, then, between most of the host species recorded is their fish-eating habit and for this reason the main search for *Renicola* was concentrated on fish-eating sea-birds."

He noted that, morphologically, members of the genus present a rather simple basic structure. Discussing specific criteria he observed (p. 5), "The characters most frequently used for the separation of the species are the linear distribution of the vitelline glands, the length of the intestinal ceca, the relative size and shape of the gonads, the sizes of the suckers and the ratio between these sizes, and egg-size.

"Of these criteria the size and shape of the gonads can be dismissed at once since they are so dependent on the state of maturity of the worms. Even the position of the testes with relation to one another and to the ventral sucker will be shown to be variable. The sizes and size-ratio of the suckers are reasonable characters provided that all of the specimens are fixed by the same technique. Pressure alters the sizes slightly and has more effect on the oral than the ventral sucker, thereby rendering the size-ratio valueless. The length of the intestinal ceca is useful but, since in most cases there are only two alternatives, either to the approximate level of the ventral sucker or more or less to the posterior end of the body, it is a character of only limited application. The linear distribution of the vitelline glands is the principal feature on which Dollfus (1946) based his key to the species of the genus. This is a useful criterion but these organs can be distorted by pressure and are undoubtedly more variable than most authors have indicated. The size of the eggs is useful in some cases but in many species the size-ranges overlap within a narrow field."

Despite his reluctance to determine specific criteria in the genus *Renicola*, Wright (1957) described *R. goliath* from two worms found in a cyst in the kidney of *Ardea goliath* taken in the southern Sudan and arranged the species of *Renicola* in four groups: (1) the *pinguis* group, in which the vitelline follicles are often large and coarse and disposed lateral to the intestinal ceca; (2) the *mediovitellata* group with dorsal vitelline follicles in linear series between the ceca; (3) the *pelecani* group, represented by a single species, *R. pelecani*, characterized by a long esophagus; and (4) the *goliath* group, also based on a single species, in which the vitelline follicles are arranged in groups throughout the midregion of the body, ventral to the ceca, from the lateral margins almost to the midline.

Wright (1956) also reported experiments designed to discover the life-cycle of species of *Renicola*. The work followed the suggestion made by Wright (1953) of a relationship between the adult trematodes and the "Rhodometopa" group of cercariae. *Cercaria rhodometopa* was described by Pérez (1924) from the genital gland of *Turritella communis* from Roscoff and the Baie de Morlaix, France. Stunkard (1932) studied the species from a single crushed snail and from a series of sections provided by Professor Pérez at the Station Biologique de Roscoff. He supplemented the specific description, worked out the flame-cell formula, and reported that the excretory vesicle is Y-shaped with lateral branched evaginations that extend throughout the body. He stated (p. 326), "The excretory system is aparently well developed and probably has already attained the definitive form which will persist through all succeeding stages in the life-cycle of the species. The system is so peculiar and characteristic that it will afford a quick and certain criterion for the identification of later stages in the life-history, and may, indeed, lead to the correlation of this larva with an adult trematode having the same excretory pattern."

Rothschild (1935) described four new species of rhodometopous cercariae from 541 specimens of *T. communis*, dredged from the Rame Mud at Plymouth, England, and two additional species from 180 specimens of the same host taken at Naples, Italy. Miss Rothschild reported that 8% of the snails at Plymouth and 5% of those taken at Naples were infected. The species from Plymouth were named *C. pythionike*, *C. doricha*, *C. herpsyllis*, and *C. nicarete;* the neapolitan species were described as *C. ampelis* and *C. ranzii*. The diagnostic features of *C. rhodometopa* and the six new species were portrayed in tabular form. The flame-cell pattern was worked out for each species and a postacetabular transverse vessel was found extending between the lateral arms of the excretory vesicle. *Cercaria doricha* differed from all the others in having 6 instead of 5 flame-cells in each of the twelve groups on each side of the body and in the presence of lateral penetration glands in addition to the medial ones that were found in all the other species. It was somewhat larger than any of the others and the least active; when introduced into the bottom of a column of sea water, it did not rise more than 10 feet whereas individuals of *C. pythionike* swam to the top of a 30-foot column in about five hours. The swimming behavior differed markedly from that of the other species. Miss Rothschild referred to the stages in the snail as Parthenita, a term introduced by Sinitsin (1911) and strongly endorsed by Faust (1918) but which is now discredited. They were designated as sporocysts, but the description contained the statement (p. 162), "Poorly developed pharynx situated anteriorly similar to that described by E. C. Faust (1921) for *Cercaria pekinensis*, but surrounded by secretory cells resembling 'salivary' glands of rediae.

Pharynx acts also as birth pore." A pharynx was not present in young sporocysts, but, according to the account, the structure was formed when a sporocyst became constricted and divided by transverse fission; whereupon each of the severed ends invaginated and formed the future "pharynx."

An intensive and assiduous search for the metacercarial stages of these larvae was conducted. According to Miss Rothschild, *T. communis* lives at a depth of 20 fathoms or more; it has a patchy and circumscribed distribution in the Plymouth neighborhood and is virtually restricted to a few areas in the region. The high rate of infection suggested that the metacercariae must occur commonly in some species found there, so hundreds of invertebrates, mollusks, crustaceans, polychaete annelids, sipunculids, and various echinoderms that were taken in the dredge with *T. communis*, were examined. Also examined were 13 species of the more common fishes taken in trawls in the area. Since the cercariae, on emergence from the snail, rise with alternate short periods of upward swimming and passive sinking and ultimately creep about on the bottom, there was little to indicate the next host. Although the search for metacercariae was fruitless and all infection experiments failed, the behavior of the cercariae suggested that the second intermediate host may be a fish. Miss Rothschild reported that development of the sporocysts was slow, since dissection of a snail that had been kept in the laboratory for six months harbored a very young infection. The undamaged state of the gonad and digestive gland of the host proved this infection to be a first generation of daughter sporocysts, and the slowness adds to the difficulty of work on the life-history.

The hypothesis that fishes serve as second intermediate hosts was confirmed by Rothschild and Sproston (1941). Miss Sproston found two of six specimens of *Gadus luscus* taken 3 September, 1940 at the Rame Mud harbored, respectively, about 100 and 2, oval, thick-walled cysts in the mesenteric tissue connecting the pyloric ceca. Also, 2 of 50 specimens of *Gadus merlangus* from various localities, each contained 3 apparently similar cysts in the same site. These cysts contained active metacercariae, little changed from rhometopous cercariae, although the species was not determined. Since the molluscan host is found at a depth of 20 fathoms or more, and yet is commonly infected with rhodometopous cercariae, these authors regarded it as exceedingly unlikely that the final host is a bird and suggested that the definitive hosts are fishes.

Other confirmatory evidence was provided by Timon-David (1953) who reported metacercariae from the pyloric ceca and mesenteries of sardines taken in the Mediterranean. The worms clearly were encysted rhodometopous cercariae and Timon-David regarded them as probably *Cercariae rhodometopa*.

Discussing interrelationships and taxonomy of the digenetic trematodes, Stunkard (1946) noted that Yamaguti (1939) had described the excretory vesicle of two species in the genus *Renicola* and that the form of the vesicle is virtually identical with that of *Cercaria rhodometopa*. Stunkard stated (p. 153), ". . . there is presumptive evidence that *Cercaria rhodometopa* is the larva of a species of *Renicola*, possibly the unnamed one reported by Dollfus (1939)." Wright (1953, 1954b) reported on a study of renicolid trematodes from birds in British zoos. Since Campbell and Sloane (1943) had found such worms in penguins hatched in the zoo, it seemed probable that the species was likely to be found in British seabirds. A search yielded about ten specimens, similar to those from the penguins, in the kidneys of a common guillemot, *Uria aalge;* the first record of a *Renicola* from a wild bird in Great Britain. Wright (1953) observed similarity between the excretory vesicle of young adults of this species and that of cercariae of the Rhodometopa group, and expressed the hope that knowledge of the larval stages may help to establish more definitely the true systematic position of this somewhat disputed genus.

In his account on the life-history and ecology of renicolid trematodes, Wright (1956) reported on the examination of about 1400 *T. communis* collected at different locations along the British coast. He found three of the species described by Rothschild (1935); at Plymouth he recorded 6.60% infection by *C. doricha* and 0.27% infection by *C. pythionike;* at Millport he recorded infections of 1.85% by *C. doricha*, 1.48% by *C. pythionike* and 0.37% by *C. nicarete*. In addition, at Millport he reported 4.20% infection by a new species, *C. doricha-pigmentata*. He described a new species, *C. cooki* which was present in 4% of the snails at St. Bride's Bay and 16.52% of those at South Haven. He reported on habitat and feeding habits of *T. communis* and noted that female snails are rarely infected. Eggs of *Renicola* spp. are fully embryonated when passed but did not hatch in fresh, brackish or sea water. The larvae emerged when the eggs were ingested by the snails and miracidia were found in the stomach and intestine; also eggs with open opercula were observed. Attempts to infect snails by feeding eggs of the parasite were inconclusive; what appeared to be primary sporocysts were observed on the intestinal wall, but they were not present more than three days after exposure. It was suggested that they may have migrated to other locations, but the evidence is not convincing.

Wright described the histology of daughter sporocysts and the cellular organization of the body-wall as the sporocysts grew. Miss Rothschild (1935) had described five layers in the wall of the sporocyst, but Wright suggested that the two outer layers are detachable and may represent a "paletot," derived from the mother sporocyst and not a part of the daughter. He described and figured a pharynx in the sporocyst and confirmed the report of Rothschild that it served as birth-pore. He stated that the pharynx had been observed in the process of formation in young specimens, differentiating from a thickened patch of "fourth layer" cells, in contrast to the suggestion by Rothschild that the pharynx arises at the locus where a sporocyst had divided by transverse fission. But in many sporocysts no trace of a pharynx could be found. Although Wright did not witness transverse division in sporocysts, he reported constrictions of circular muscles which produced what appeared to be chains of sporocysts.

In addition to his study of miracidia removed from the intestine of snails that had fed on renicolid eggs, and of larval stages from naturally infected hosts, Wright (1956) reported on experiments conducted to obtain infections in the laboratory. He noted that it is impossible to raise *T. communis* from egg beyond the late veliger stage; consequently, the snails used for the experiments were collected in the field. It is exceedingly difficult to determine whether or not such snails are free of trematode infection. Under laboratory conditions, infected snails may cease to liberate cercariae and recently established infections may fail to develop or may remain inactive for long periods, with no indication of their presence, as reported by Miss Rothschild (1935). The snails used by Wright were tested by isolating them in 25 by 75 mm. glass tubes and raising the temperature to 15° C., which may induce shedding, but failure to liberate cercariae is no guarantee that the specimen is uninfected. Three attempts at infection were reported. In the first, 42 snails, tested for a period of 4½ months, were exposed overnight to eggs of *Renicola* sp. taken from *Colymbus arcticus*. Weekly and fortnightly dissections of snails were negative, but one female with a shell length of 35 mm., which was dissected at the end of 3½ months, contained "very immature sporocysts in the gonad." In the second experiment, 69 snails, previously tested for 4 months, were exposed overnight to eggs of *Renicola* sp. taken from *P. puffinus*. Snails were dissected at fortnightly intervals for 3½ months, when the last snail died. No infections were found. In the third experiment, 60 snails, previously tested for 2½ months, were exposed for six hours to eggs of *Renicola* sp. taken from *P. puffinus*. Fortnightly examinations for 3½ months gave only negative results and, at the end of 6 months, the 8 remaining specimens were dissected. Four contained sporocysts, somewhat more advanced than those found in the snail from the first experiment.

Wright (1956) also reported attempts to infect birds. He found heavy infection of *Clupea sprattus* by two different types of cysts, one form larger and more rounded than the other, which had an elongate-oval shape. The first kind was found to contain larvae of the *C. doricha* type and the second contained larvae of the *C. pythionike* type. Cysts of both types were fed to a domestic duckling and a domestic chicken, both about six weeks old, and an adult blackbacked gull. The duckling and the chicken each received 300 cysts and the gull received 1700 cysts, but no infections were found.

The morphological similarity of rhodometopous cercariae and young renicolid trematodes from the renal tubules of birds is striking and the repeated failures to infect either snails or avian hosts was baffling.

Prospectus and Procedures

The present investigation was begun June 5, 1958. At the time, the writer was engaged in a study of the parasites and predators of *Mya arenaria* for the U. S. Fish and Wildlife Service. At Sam's Cove, near Boothbay Harbor, Maine, a portion of the cove had been fenced to exclude green crabs, *Carcinus maenas*. At one side of the cove there was an extensive bed of *Mytilus edulis* with enormous numbers of drills, *Thais lapillus*, feeding on the mussels and on the barnacles which grew on the mussel shells. Some 400 of these *T. lapillus* were collected and examined; one of them shed an undescribed cercaria. The cercaria was studied and the snail was crushed to obtain the asexual generations of the parasite. No other infection by this species was observed until 1961, when it was found again in *T. lapillus* taken on the north shore of Cape Cod, Massachusetts.

Only two species of trematode larvae are known from *T. lapillus*. In addition to the newly-found cercaria, this snail harbors the asexual generations of *Parorchis acanthus*. The latter species is common, infecting from one to three per cent of the snails in the New England area. It occurs also in *Urosalpinx cinerea* of New England and in *Thais floridana* at Pensacola, Florida. Its life-cycle was worked out by Stunkard and Cable (1932); these authors identified the adult as *Parorchis* *avitus* (Linton, 1914), later recognized as identical with *Parorchis acanthus* (Nicoll, 1906) Nicoll, 1907.

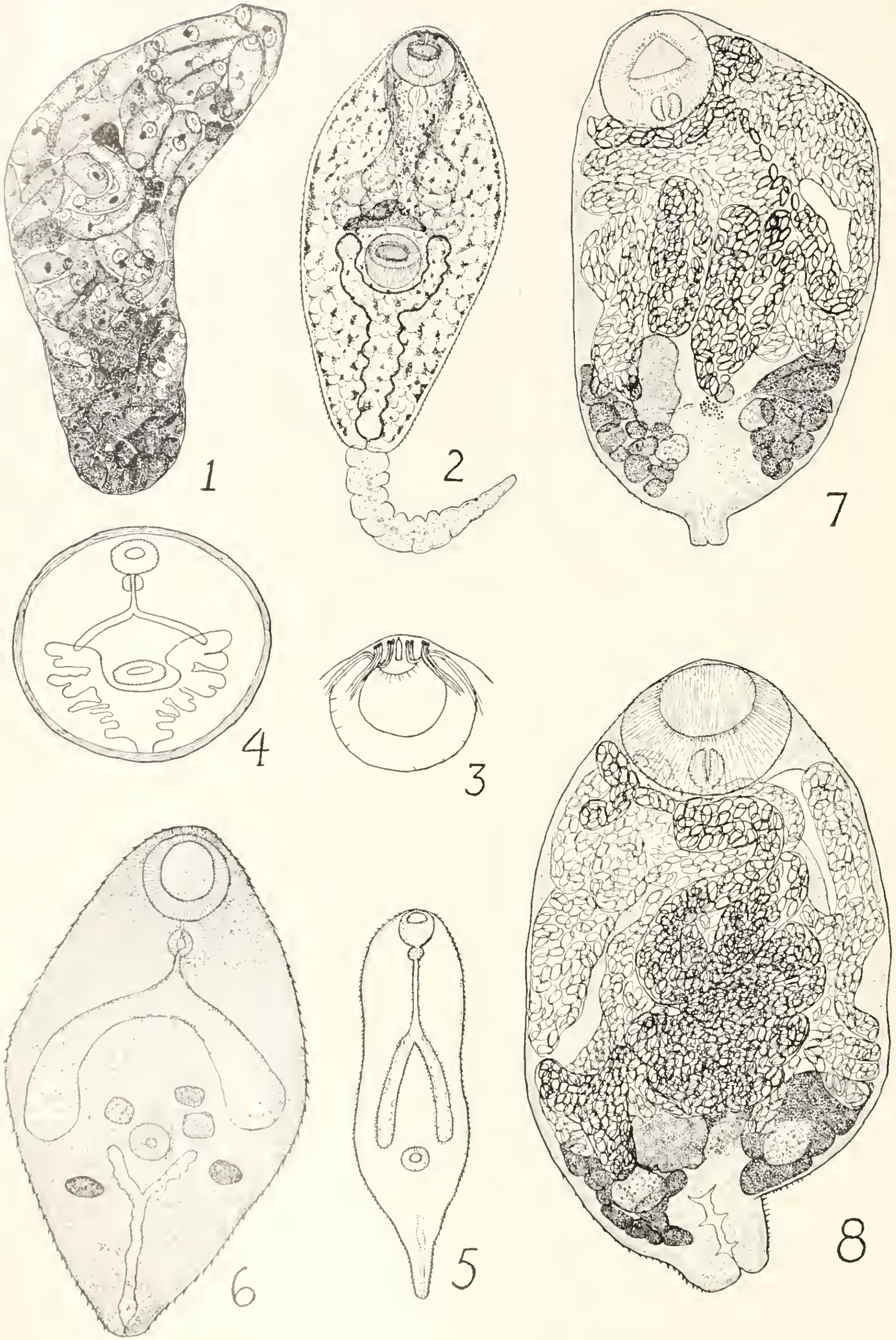
1961

During the summer of 1961 over 1500 specimens of *T. lapillus* were collected and examined in a search for the undescribed cercaria. The snails were isolated, five in a fingerbowl; the water was checked and changed morning and afternoon each day, for five days. At the end of that period, representative samples, one of ten or one of twenty-five, were crushed and examined for recent infections and the remainder were discarded. Three infections were found: one of 340 snails collected June 21; one of 408 snails collected June 30; and one of 324 snails collected July 13. The specimens from which cercariae emerged were kept until they died, in order to have cercariae for experiments on their life-cycle. One snail lived only 10 days, another 18 days, the other about two months. It is probable that the warm summer temperature of the water in the Marine Biological Laboratory, where the work was done, had a deleterious effect on the infected snails. At first they shed profusely, thousands of cercariae in 24 hours, but the number diminished rapidly and after about two weeks only a few cercariae emerged each day. During the summer, hundreds of invertebrates and vertebrates, including turbellarians, nemerteans, annelids, echinoderms, crustaceans, mollusks, and fishes were exposed to the cercariae. A stream of cercariae was sucked into the incurrent siphon of *Mytilus edulis*, and *Pecten irradians* was especially susceptible, drawing the larvae into the gill area with each pulsation. The larvae encysted and persisted only in bivalve mollusks, especially *Mytilus edulis*, *Pecten irradians*, and *Gemma gemma*. They did not form metacercariae in *Mya arenaria*, or in the oyster, *Crassostrea virginica*.

The metacercariae did not grow in their cysts, and, after allowing several days for them to attain maturity, they were fed to mice, hamsters, laboratory-raised gulls, *Larus argentatus*, and efforts were made to feed them to fishes. But the fishes, except for cunners, *Tautogolabrus adspersus*, and species of *Fundulus*, refused to eat despite various devices hopefully contrived. The apparent northern distribution of the species suggested cold-water fishes, *e.g.*, the wolf-fish, *Anarchis lupus*, or the eel-pout, *Zoarces anguillaris*, both of which feed on mollusks, echinoderms and crustaceans, might harbor the sexually mature stage. But at the end of the summer, the final hosts were quite unknown.

1962

The study was resumed at Woods Hole in the summer of 1962. Between May 23 and August 23, over 2600 *T. lapillus* were isolated and six of them liberated cercariae. One snail, dissected June 13, had a recent infection, but was not yet shedding cercariae. The first shedder was found June 18 after the isolation of 940 snails. A second shedder was found on June 23, from 220 snails taken in the same area as the last collection. It was now clear that the infection was concentrated in particular places, and snails taken from other locations were not infected. Specimens of *M. edulis*, exposed to cercariae from these snails since June 20, were fed on June 29 to two baby domestic chicks and two baby gulls, *Larus*



All figures are of *R. thaidus*

argentatus. The birds were fed on processed canned food, supplemented by multiple vitamins, since the natural accessory food-factors are diminished or destroyed in processing. On July 4, the chicks were necropsied; examination of the tissues was done in a solution of one-half sea water. No worms were found. On July 5, the two baby gulls were sacrified and three very small but active worms (Fig. 5) were found in the kidneys of each bird. Actually the worms were found in the fluid in which the kidneys were being or had been dissected. The worms agreed morphologically with the metacercariae fed six days earlier.

Since it appeared that the species was a renal parasite of birds, the experiment was extended. On July 6, three young gulls, now older and more robust, were fed metacercariae, encysted in *M. edulis* and *P. irradians.* On July 11, two domestic mallard ducklings and two half-grown domestic chickens were fed encysted metacercariae. On July 18 a young gosling was fed metacercariae and, in the ensuing weeks, the birds were fed metacercariae at intervals as they became available in *M. edulis, P. irradians,* and *G. gemma,* but *M. edulis* and *P. irradians* were employed chiefly since they accumulated the metacercariae more readily and in greater numbers. On July 29, one of the gulls exposed on July 6 was sacrificed and three worms (Fig. 6) were found in the kidneys. These worms were motionless and had to be dug out of the tubules; they were delicate and blistered easily. Between July 31 and August 22, both chickens, both ducks, the gosling, and one gull were autopsied and no worms were found. But on August 23, necropsy of the last gull yielded seven mature worms in the kidneys. They are clearly members of the genus *Renicola,* thus representing the first experimental infection by a member of the genus. To distinguish these specimens from others, they are designated as a new species, *Renicola thaidus.* The two specimens shown in Figures 7 and 8 are deposited in the Helminthological Collection of the U. S. National Museum, under the number 60,762. The larger worm (Fig. 8) is designated as type of the species.

The work at Woods Hole was discontinued on August 27, when the writer left for Vancouver, Canada, to join the Faculty of the University of British Columbia for the first semester of the academic year, 1962–1963. There, Dr. Hilda Ching stated that she had found a cercaria, very similar to, and possibly identical with, the renicolid species of New England, in *Thais emarginata, Thais lamellosa,* and *Searlesia dira* taken at Friday Harbor, Washington. *Thais emarginata* and *Searlesia dira* do not occur at Vancouver and the examination of 760

Plate I

Figure 1. Sporocyst, from *Thais lapillus;* from a fixed and stained specimen; 0.86 mm. long.

Figure 2. Cercaria, drawn from pencil sketches of living and fixed specimens.

Figure 3. Cercaria, anterior end to show openings of penetration glands and position of stylet, from pencil sketches of living specimens.

Figure 4. Metacercaria, from *Mytilus edulis,* from pencil sketches of living specimen.

Figure 5. Juvenile worm, three days in gull, 0.025 to 0.045 mm. long and 0.08 to 0.13 mm. wide; from pencil sketches of living specimen.

Figure 6. Juvenile worm, three weeks in gull, 0.68 mm. long, much flattened, fixed and stained specimen.

Figure 7. Adult specimen, 0.84 mm. long, fixed and stained, ventral view.

Figure 8. Adult specimen, 0.97 mm. long, fixed and stained, ventral view.

specimens of *T. lamellosa* taken from rocks off Stanley Park did not disclose infection with the cercaria.

1963

The experiments of 1962 were disconcerting, since after feeding thousands of metacercariae, so few worms were recovered. Although the worms matured in the renal tubules of a gull, it appeared that the gull is not the natural host and that some other bird, perhaps one of the diving ducks that feed on mussels, would be more susceptible to infection. Belopolskaia (1952) described *Renicola somateriae* from *Somateria spectabilis*, and Kulachkova (1957) described *Renicola mollissima* from *Somateria mollissima*. Accordingly, at my request, Mr. Walter R. Welch of the staff of the U. S. Fish and Wildlife Service at Boothbay Harbor, Maine, and his associates collected eggs of the eider duck, *Somateria mollissima*, and hatched the chicks in the laboratory there. The investigation was resumed at Woods Hole on May 16, 1963. Collection and isolation of *T. lapillus* was continued but none of the first 2000 snails shed cercariae. A trip was made July 3 to Boothbay Harbor to get the eider chicks. There, three adult birds, *Somateria mollissima*, were autopsied; the intestine was filled with finely ground shells of *Mytilus edulis* but no kidney-flukes were found. Also, about 1000 *T. lapillus* were collected at Sam's Cove, where the first infection had been found in 1958. None of these snails shed cercariae nor any of a shipment of 576 sent by Walter Welch on August 13. The return to Woods Hole with the eider chicks and snails was made on July 5.

The same afternoon an airplane shipment was received from Dr. Hilda Ching in Vancouver, British Columbia. It contained 11 specimens of *Thais emarginata*, 98 of *T. lamellosa* and 19 of *S. dira*, but the material had been delayed in transit and only two *T. emarginata* were alive. They lived for only two or three days and were not infected. The time when a snail dies is difficult to determine; it may be alive, but inactive, or may respond to stimuli when moribund. On July 25, a second shipment, consisting of 301 *T. lamellosa* was received from Dr. Ching. The snails had been put on a plane in Vancouver at 8:00 PM, July 24, and arrived at Hyannis airport at 2:05 PM on July 25. At 3:00 they were in sea water at the Marine Biological Laboratory, but the water was warm and the snails remained sluggish. Two of them shed renicolid cercariae; one lived but 2, and the other only 4, days. Specimens of *M. edulis* and *P. irradians* were placed in fingerbowls with the cercariae from these snails, July 27 to July 31. On August 6, examination of these bivalves showed many metacercariae and on August 6 and August 8, the metacercariae were fed to a young gull and to two eider ducks, A and B. A third shipment from Dr. Ching was received on August 9, but this shipment had been delayed and did not go to the Hyannis airport; instead it was delivered by truck on the afternoon of the following day and all the snails were dead.

The first infection of *T. lapillus* was found on July 28, when one of 360 specimens shed cercariae. Another collection on August 6, of 582 snails from the same area, yielded four shedders and a third collection of 564 snails on August 9, yielded five shedders. The infected snails do not survive well in the laboratory, but provided thousands of cercariae used for infection of *M. edulis* and *P. irradians*. Eider ducks C and D were fed metacercariae on August 6, August 12, and August 19. On August 12, August 19, and subsequently, eider ducks E and F were fed large numbers, hundreds, of metacercariae encysted in *M. edulis* and *P. irradians.* Eider duck A, fed metacercariae derived from *T. lamellosa,* August 6 and August 8, was autopsied on August 22, and no worms were found. Eiders C and D, fed August 12 and August 19 metacercariae derived from *T. lapillus,* were autopsied on August 26 and August 28, and no worms were found. Eiders E and F, also fed metacercariae from *T. lapillus,* were autopsied on August 30 and September 24, respectively, with negative results. Eider B, fed metacercariae from Vancouver snails, was autopsied September 29 and no worms were found. The gull, also fed August 6 with metacercariae from *T. lamellosa,* was autopsied on October 4, but no worms were recovered.

The cercariae from *T. lamellosa* were the same size as those from *T. lapillus* and no morphological differences were observed. Whether or not the cercariae which occur in *T. emarginata, T. lamellosa* and *S. dira* of the Pacific coast are identical with those from *T. lapillus* of New England can not be determined from data now available.

The studies of Wright and Timon-David had implicated the rhodometopous cercariae as larvae of species in the genus *Renicola.* The observations of Stunkard (1932) on *Cercaria rhodometopa* were made on living material and on a series of sections loaned by Professor Pérez. Accordingly, request was made to Dr. Wright for a loan of specimens of a rhodometopous species for comparison with the cercariae from *T. lapillus.* He kindly agreed and sent the infected gonads of two *Turritella communis* which were received on May 22, 1963. The material was sectioned but apparently the snails were dead when fixed and the sporocysts and cercariae did not stain well.

At this time it is a pleasure to acknowledge the indebtedness to Mr. Walter R. Welch, to Dr. Hilda Ching, and to Dr. C. A. Wright for the generous contributions they have provided; my grateful appreciation is herewith expressed.

After eiders C, D, E, and F had received heavy feedings of metacercariae, the cercariae each day were placed in well-aerated aquaria with small fishes, to determine whether or not they would encyst in the wall of the intestine or its adnexa, as reported by European authors in the case of the rhodometopous cercariae. Members of the following species were exposed: *Fundulus heteroclitus, Menidia menidia, Gasterosteus aculeatus, Clupea harengus, Osmerus mordax, Tautogolabrus adspersus,* and *Paralichthys dentatus.* Dissection of these fishes later did not disclose encysted metacercariae and apparently they do not serve as natural intermediate or paratenic hosts.

When released normally from *T. lapillus,* mature cercariae are filled with cystogenous-gland cells and the details of the excretory system are hopelessly obscured. Since so few infected snails were found and the cercariae were required for experimental infections, no shedding snails were sacrificed in 1961 or 1962. Dead or moribund snails were crushed, but the sporocysts and cercariae were also dead or moribund, and attempts to resolve the flame-cell pattern were futile. In 1963, after the infection of bivalve mollusks was ended and the fishes had been exposed to cercariae, the one remaining shedding snail was crushed on September 18, to obtain sporocysts and developing stages of the cercariae for study of the excretory system.

DESCRIPTIONS

Sporocysts

Primary or mother sporocysts have not been observed. Daughter sporocysts (Fig. 1) fill the body of the snail (Fig. 9), replacing the gonad and almost all of the digestive gland. The intensity of the infection, with the destruction of host tissue, is undoubtedly a prime factor in the early death of infected snails brought into the laboratory. Deprived of food, and with elevated temperature, these specimens have little reserve or resistance.

The sporocysts are spherical to oval to pyriform to cylindrical, with rounded ends. Sometimes annular constrictions produce a dumb-bell shape or the protrusion of one or both ends. Often the anterior end, which bears the birth-pore, is extended as a result of the activity of cercariae in the region. The sporocysts vary greatly in size, measuring from 0.3 by 0.2 mm. to 1.26 by 0.40 mm. When removed from the snail, the sporocysts may be embedded in fibrous tissue which closely invests them and binds them together. This tissue is probably of snail origin, derived from the vascular fluid in which the sporocysts are immersed. In addition, each sporocyst has a two-layered wall, and the two layers are often separated in sections (Fig. 10) of the snail tissue. The inner layer is thin and delicate while the outer layer is thicker and stains more heavily. At interstices between sporocysts, the outer layer becomes continuous with the fibrous tissue in which the sporocysts are embedded. If the external layer is actually a "paletot," derived from cells of the mother sporocysts, these cells must have enormous multiplicative capacity to be able to envelop all the daughters. To build up such an overwhelming infection, it is likely that daughter sporocysts produce and liberate daughters before they begin to produce cercariae. The wall of the sporocyst is thin and, when stretched by pressure of moving cercariae, it is almost membranous. But movements of the sporocyst show that the wall contains both longitudinal and circular muscle fibers, although their action is slow and feeble. At the end which bears the birth-pore there is a sphincter, a band of circular fibers associated with diagonal fibers that are inserted in the body wall, which closes the opening after a cercaria has emerged. This muscular development may simulate a pharynx, but the sporocyst has neither pharynx nor digestive sac. Blood of the snail may enter the sporocyst through the birth pore. The germinal cells, that produce the ensuing generation, are free in the cavity of the sporocyst. They multiply by polyembryony and produce germ-balls that differentiate and develop into cercariae. The cercariae emerge from the sporocysts and progress by way of the vascular system to the gills of the snail, from which they escape into the sea water. The liberation of hundreds of cercariae each 24 hours probably produces lesions in the gills that hasten the death of the snail.

Cercaria

The cercariae (Fig. 2) are oval to pyriform, and either end may be wider as the larva moves. They are flattened dorsoventrally, the musculature is very slight, and, under a coverglass, the cercaria blisters easily. The body is 0.25 to 0.38 mm. long and 0.06 to 0.13 mm. wide. The tail is shorter than the body; ordinarily it is less than one-half body length and may measure 0.08 to 0.13 mm. long and 0.029

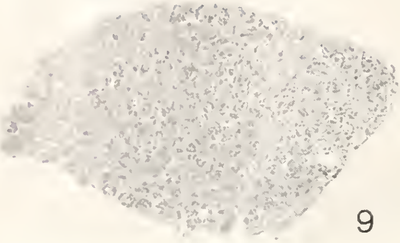
to 0.036 mm. wide at the base, but the tail may be extended to a length of 0.24 mm. In swimming, the body is contracted, bent ventrally, while the tail is extended and lashes vigorously. The larvae are not strong swimmers. The acetabulum is slightly behind the middle of the body and measures 0.038 to 0.043 mm. in diameter. The cuticula of the body is armed with sharp-pointed spines that become smaller and apparently more sparse posteriorly. There is a staggered double row of larger spines, about 100 in number and 0.003 mm. in length, around the openings of both the oral and acetabular suckers. The anterodorsal wall of the oral sucker bears a hastate stylet, 0.008 to 0.010 mm. in length. The oral sucker is the same size as the acetabulum; there is a very short prepharynx, visible only when the anterior end is extended; and the pharynx is 0.019 to 0.021 mm. in diameter. The anterior part of the esophagus is the only other part of the digestive tract to be recognized.

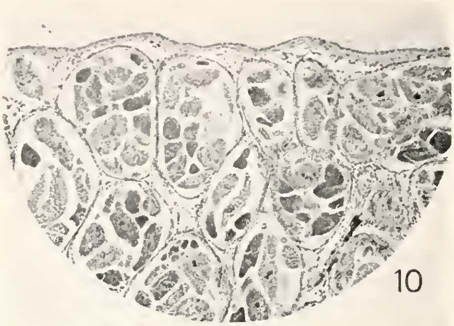
There are five pairs of penetration glands, situated in the preacetabular area. Their ducts pass forward in two bundles lateral and dorsal to the oral sucker. The lateral bundle has two ducts which open medial and ventral to the dorsolateral openings of the three medial ducts (Fig. 3). Cytogenous glands, mostly dorsal in position, fill the parenchyma of the body. They measure 0.014 to 0.015 mm. in diameter when viewed from above and from side view they appear flask-shaped, 0.028 mm. in length and 0.008 mm. in width at the narrow end. Interstices between the glands are filled with refractile spherules, 0.001 to 0.003 mm. in diameter.

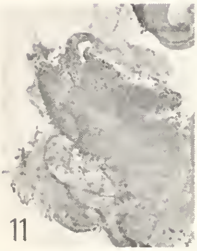
The excretory system was studied in cercariae from the snail crushed on September 18, 1963. Germ-balls have two flame-cells and two posterolateral excretory pores. The posterior parts of the two primary tubules fuse to form the stem of the excretory bladder. At the anterior end of the stem, anterolateral protrusions form the arms of the definitive bladder which extend forward on either side of the acetabulum. In young cercariae, collecting ducts discharge into the bladder at the sides, near the anterior end of the stem of the vesicle. These ducts extend forward to the acetabular level where they divide into anterior and posterior branches. Each branch receives the capillaries of three flame-cells and the early pattern is $2\ [(1+1+1)+(1+1+1)]$. Later, additional flame-cells appear and the definitive formula is $2\ [(3+3+3)+(3+3+3)]$ flame-cells. The wall of the bladder is epithelial, with deeply staining nuclei, but it is very flexible, and bulges appear repeatedly at different locations. There is a strong posterior sphincter, with lateral excretory pores at the junction of the body and tail. The organization of the excretory system is strikingly similar to that of *Cercaria parvicaudata* as reported by Stunkard (1950; Figs. 1–4).

Metacercaria

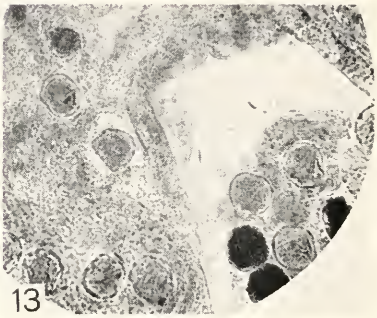
The cercariae encysted in enormous numbers in the gills, walls of the suprabranchial chambers, mantle and foot of *Mytilus edulis* (Figs. 11, 12, 13) and *Pecten irradians* (Figs. 14, 15, 16). The metacercariae (Fig. 4) are spherical to oval, depending on pressure from the surrounding tissues. They measure 0.12 to 0.16 mm. in diameter and the wall of the cyst is 0.008 to 0.009 mm. in thickness. The larvae do not grow in the metacercarial stage and cysts do not increase in size.

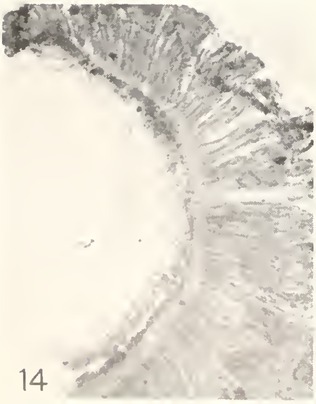

9

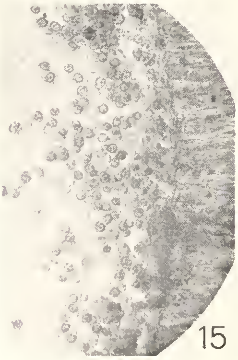

10


11

12


13


14


15

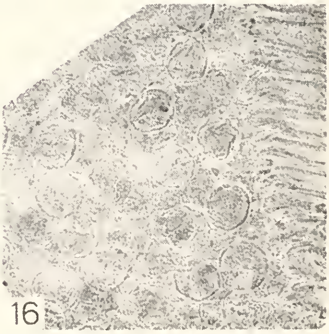

16

Plate II

All figures of unretouched photographs

FIGURE 9. Portion of a section of *Thais lapillus*, to show intensity of infection; fixed and stained specimen.

FIGURE 10. Part of section shown in Figure 9, enlarged.

Adult

Sexually mature and gravid specimens (Figs. 7, 8) are oval to pyriform, 0.70 to 1.16 mm. in length and 0.40 to 0.60 mm. in width. They are rounded anteriorly, somewhat attenuated posteriorly but the body does not extend far behind the vitellaria. When retracted, the posterior end may form a knob-like protuberance. The cuticula is armed throughout with sharp, distinctly separated spines. The musculature is delicate and very weak. When the body is congested with uterine coils that are filled with eggs, movement is feeble and slow, limited to changes in form. The acetabulum is situated at the testicular level, immediately posterior to the genital pore. It is not visible in either of the specimens shown on Plate I, but in another, crushed specimen it is 0.02 mm. in diameter. The mouth is subterminal. The oral sucker is 0.26 to 0.30 mm. in diameter; the wall is loosely organized, spongy and vacuolated in appearance. There is no prepharynx and, in fixed and stained specimens, the pharynx, which measures 0.052 to 0.060 mm. in diameter, is embedded in the wall of the oral sucker. There is no esophagus and the digestive ceca extend to the testicular level. The excretory pore is terminal and the vesicle is Y-shaped; the bifurcation is post-testicular; the wall is very thin and pliable with lateral evaginations wherever pressure is relaxed. Masses of eggs obscured the arms of the vesicle and lateral evaginations were not observed.

The reproductive organs are compressed into a small region near the posterior end of the body. The testes are opposite, almost spherical, 0.06 to 0.09 mm. in diameter, situated about one-fifth of the body length from the posterior end. Other parts of the male system were not observed, but there is no reason to believe that they are essentially different from those in other species and described by Stunkard, Nigrelli and Gandal (1958). The ovary is dextral, lobed, slightly anterior to, but extending into, the testicular zone. It is elongate, 0.16 to 0.24 mm. in length and 0.12 to 0.16 mm. in width. The structures of the oötype region are so compacted that details are not clear. Attempts to make serial sections of worms with such flimsy and weak musculature and such masses of eggs are usually fruitless and were not undertaken. The vitellaria consist of 16 to 18 oval to lobed to clavate follicles, situated on each side of the body, overlapping the testicular zone both anteriorly and posteriorly, and partially overlapping the testes on the dorsal side of the body. The follicles measure 0.02 to 0.07 mm. in diameter; in pressed specimens when follicles are superimposed, their outlines may be indefinite. The uterus passes forward from the oötype, forming numerous irregular loops and coils which fill the body in front of the gonads. Young eggs have clear, almost transparent, shells and the contents stain faintly; as they pass along the uterus the shells become thicker and darker in color. Eggs are operculate and in mounted specimens measure 0.036 to 0.038 mm. long and 0.016 to 0.017 mm. wide.

FIGURE 11. *Mytilus edulis*, portion of a crushed individual, showing metacercariae of experimental infection; fixed and stained specimen.

FIGURE 12. Portion of Figure 11, enlarged.

FIGURE 13. Portion of Figure 12, enlarged.

FIGURE 14. *Pecten irradians*, portion of a gill, showing metacercariae of experimental infection; fixed and stained specimen.

FIGURE 15. Portion of Figure 14, enlarged.

FIGURE 16. Portion of Figure 15, enlarged.

They are embryonated when passed, and when measured with living miracidia they averaged 0.042 by 0.020 mm.

DISCUSSION

The cercariae from *Thais lapillus* and *Thais lamellosa* are very similar to *Cercaria parvicaudata* Stunkard and Shaw, 1931 from *Littorina littorea*, *Littorina saxatilis* and *Littorina obtusata* taken at Woods Hole, Massachusetts, and *Cercaria roscovita* Stunkard, 1932 from *Littorina saxatilis nigrolineata* taken on the Brittany coast near Roscoff, France. The resemblance is striking, particularly in the development and structure of the excretory system as reported for *C. parvicaudata* by Stunkard (1950). Referring to *C. parvicaudata* and *C. roscovita*, Stunkard (1950: p. 142) stated, "The general structure of the two species, and especially the details of the excretory system, suggest that they are not only closely related but that they are probably members of the family Plagiorchiidae. Since members of this family are rare or unknown in marine fishes, and occur most frequently in birds and mammals, it is possible that some bird or mammal which frequents the sea-shore is the final host of *Cercaria parvicaudata*." The final host of *C. parvicaudata* is still unknown, but the discovery of the life-cycle of *R. thaidus* raises important taxonomic problems.

In the classification of La Rue (1957), *Renicola* was named type of not only a family, but of a superfamily and an order, Renicolida, in the Anepitheliocystidia, a major group of the digenetic trematodes in which the wall of the excretory bladder is not epithelial. The allocation by La Rue was based on the belief that *Cercaria rhodometopa* is a larval stage of a species of *Renicola*. This belief arose from, and was supported by, the unusual and characteristic form of the excretory bladder, which is Y-shaped and extends the length of the body and in which the stem, as well as the arms, are extensively branched. Such a feature appeared characteristic of the rhodometopous cercariae and of the adults of renicolid species. The observations of Rothschild and Sproston (1941), Timon-David (1953) and Wright (1953, 1956), that metacercariae encysted in the pyloric ceca and adnexa of the digestive system of fishes are morphologically similar to the renicolid cercaria, and the reports by Wright on the agreement of these metacercariae with juvenile worms from the kidneys of birds, appeared to confirm the life-cycle, despite the failure of infection experiments.

The differences between *C. rhodometopa* and *C. thaidus* are conspicuous. *Cercaria rhodometopa* lacks a stylet; it has a large and powerful tail provided with fins; and the flame-cell formula of the excretory system is $2\,[(5+5+5+5+5+5)+(5+5+5+5+5+5)]$. The material sent by Dr. Wright is so impaired and disorganized that it is not possible to determine with certainty whether or not the wall of the excretory vesicle is epithelial. *Cercaria thaidus* has a stylet; the tail is small, without fins; and the flame-cell formula is $2\,[(3+3+3)+(3+3+3)]$. It is unlikely that species in a single genus have cercariae as radically different as *C. thaidus* and *C. rhodometopa*. The systematic relations of the rhodometopous cercariae are uncertain, but the epithelial bladder of *C. thaidus* definitely removes the genus *Renicola* from the Anepitheliocystidia, and the family Renicolidae is here included in the Plagiorchioidea, near the family Lecithodendriidae.

Comparison of the sexually mature stage of *R. thaidus* with previously described species of the genus is very unsatisfactory. As noted, especially by Dollfus (1946) and Wright (1954, 1956), the distinctions between the described species are so tenuous that it is quite impossible to characterize any species with assurance. It is reasonably certain that many of the described species are identical, but criteria for specific distinctions are obscured by multifarious individual variations. In the description of *R. cruzi*, Wright (1954) noted that the specimens differed from *R. lari* only in smaller size and smaller size of eggs. He stated (p. 63), "It may be that this species is no more than a race of *R. lari*, but this will only be shown by detailed life-history studies." *Renicola lari* Timon-David, 1933 is perhaps the best known species; Caballero y Caballero (1954) declared that *R. lari* is identical with *R. glandoloba* Witenberg, but Wright (1956) observed that the differences between the two species are as great as those employed by Caballero to differentiate the species described by him as new. The status of *R. magnicaudata* Bykhovskaia-Pavlovskaia, 1950 from the barn swallow, *Hirundo rustica*, was questioned by Wright (1956) and the species was transferred to *Cortrema* by Khotenovsky (1961). Until life-histories are known, with opportunities to measure developmental changes and individual variations, the present chaotic situation will probably continue.

Renicola thaidus is one of the smallest species and, although information for comparison is limited, it differs from all previously described forms in one or more of the following features: relative size of oral sucker, pharynx and acetabulum; length and extent of the digestive ceca; relative position and size of the gonads; location and extent of the vitellaria and size of the individual follicles; and size of eggs.

LITERATURE CITED

Belopolskaia, M. M., 1952. Die Parasitenfauna von Meereswasservögeln (in Russian). Ucenye Zapiski Leningrad. gosudarstv. Univ., 141, Ser. biol. Nauk **28**: 127–180. (Cited from Odening, 1962.)

Bykhovskaia-Pavlovskaia, Irina E., 1950. New species of kidney parasites (genus *Renicola*) of birds (in Russian). Dokl. Akad. Nauk. SSSR., n. s. 71(2): 412–417. (*Cf. Helminth. Abstracts,* **19**: 90–91.)

Bykhovskaia-Pavlovskaia, Irina E., 1962. Trematodes of birds in the USSR (in Russian). Moscow-Leningrad, 408 pp., Verlag. Akad. Wiss. USSR.

Caballero y Caballero, E., 1953. Helminths from the republic of Panama. VI. A new trematode of the family Renicolidae Dollfus, 1939. Thapar Commem. Vol., : 23–30.

Callot, J., 1946. Matériaux pour servir à la faune des distomes de France. *Ann. Parasitol.,* **21**: 199–201.

Campbell, J., and J. E. N. Sloane, 1943. On a possible new species of *Renicola* parasitic in the kidneys of the king penguins in the Edinburgh Zoo. *Veterin. Jour.,* **99**: 291.

Cohn, L., 1904. Helminthologische Mitteilungen. II. *Arch. Naturg.,* **70**: 229–252.

Dollfus, R. P., 1939. Distome d'un abcès palpébro-orbitaire chez une panthère. Possibilité d'affinités lointaines entre ce distome et les Paragonimidae. *Ann. Parasitol.,* **17**: 209–235.

Dollfus, R. P., 1946. Sur un distome du genre *Tamerlania* K. I. Skrjabin, 1924 avec un catalogue des trématodes des reins d'oiseaux. *Ann. Parasitol.,* **21**: 25–73.

Dollfus, R. P., and A. Capron, 1958. Sur une espèce monorchide du genre *Renicola* L. Cohn 1903, parasite d'un accipitriforme d'Afrique occidentale. *Ann. Parasitol.,* **33**: 232–239.

Faust, E. C., 1918. Life history studies on Montana trematodes. *Illinois Biol. Monogr.,* **4** (1): 1–120.

Faust, E. C., 1921. The excretory system in Digenea. IV. A study of the structure and development of the excretory system in a cotylocercous larva, *Cercaria pekinensis* nov. sp. *Parasitol.*, **13**: 205–212.

Ginetsinskaia, T. A., 1952. Helminths of migratory Charadriformes in the Volga delta. *In:* K. I. Skrjabin Annivers. Volume (1953): 147–156.

Gubanov, N. M., 1954. Die Helminthenfauna wirtschaftlich verwendbarer Tiere des Ochotskischen Meeres und des Stillen Ozeans (in Russian). Tez. Canad. Diss., 1952; Trudy gel'mint. Labor. Akad. Nauk SSSR, 7: 380–381. (Cited from Odening, 1962.)

Khotenovsky, I. A., 1961. Morphology and taxonomy of trematodes of the genus *Cortrema* Tang, 1951 (Lecithodendriidae Odhner, 1911), Parazitolog. Sbornik, 20: 337–338 (in Russian; *cf. Helminth. Abstr.*, **32**: 208; June, 1963).

Kulachova, V. G., 1957. Eine neue Art der Nierentrematoden *Renicola* aus *Somateria mollissima*. Trudy Leningrad Obsc. Estestvoisp. 73: 198–203 (in Russian). (Cited from Odening, 1962.)

La Rue, G. R., 1957. The classification of digenetic Trematoda: a review and new system. *Exp. Parasitol.*, **6**: 306–349.

Lumsden, R. D., and J. A. Zischke, 1963. Studies on the trematodes of Louisiana birds. *Zeitschr. Parasitenk.*, **22**: 316–366.

McIntosh, A., and M. M. Farr, 1952. *Renicola brantae* n. sp. from the kidney of the Canada goose, *Branta canadensis* (Linnaeus, 1758). *J. Parasitol.*, **38** (Suppl.): 35–36.

Nezlobinsky, N., 1926. Helminthological studies in the Okhrida valley. I. On kidney trematodes in birds. *Galsnik Tsentral Khig. Zavoda, Beograd*, **1**: 202–217.

Odening, K., 1962a. Trematoden aus indischen Vögeln des Berliner Tierparks. *Zeitschr. Parasitenk.*, **21**: 381–425.

Odening, K., 1962b. Neue Trematoden aus vietnamesischen Vögeln des Berliner Tierparks (mit einer Revision der Familie Renicolidae). *Bijdragen tot de Dierkunde, Amsterdam*, **32**: 49–63.

Pérez, Ch., 1924. Le complexe éthologique de la turritelle et du *Phascolion strombi*. *Bull. Soc. Zool. France*, **49**: 341–343.

Rothschild, Miriam, 1935. The trematode parasites of *Turritella communis* Lmk. from Plymouth and Naples. *Parasitol.*, **27**: 152–170.

Rothschild, Miriam, and Nora G. Sproston, 1941. The metacercaria of *Cercaria doricha* Roths., 1934, or a closely related species. *Parasitol.*, **33**: 359–362.

Sinitsin, D. T., 1911. La génération parthénogénétique des trématodes et sa descendance dans les mollusques de la mer noire (in Russian). *Mém Acad. Sci., St. Petersb.*, (*8*), **30**: 1–127.

Skrjabin, K. I., 1924. Nierentrematoden der Vögel Russlands. *Centrb. Bakt., 2 Abt.*, **62**: 80–90.

Skrjabin, K. I., 1947. Trematodes of Animals and Man. Les Bases de la Trematodologia. Vol. 1: 261–277 (in Russian).

Sokolova-Andronova, E. V., 1937. (Andronova, Elena Viacheslavovna.) The renal trematodes of birds (in Russian). Rabot Gel'mint. (Skrjabin), pp. 671–672.

Stunkard, H. W., 1932. Some larval trematodes from the coast in the region of Roscoff, Finistère. *Parasitol.*, **24**: 321–343.

Stunkard, H. W., 1946. Interrelationships and taxonomy of the digenetic trematodes. *Biol. Rev., Cambridge*, **21**: 148–158.

Stunkard, H. W., 1950. Further observations on *Cercaria parvicaudata* Stunkard and Shaw, 1931. *Biol. Bull.*, **99**: 136–142.

Stunkard, H. W., and R. M. Cable, 1932. The life-history of *Parorchis avitus* (Linton) a trematode from the cloaca of the gull. *Biol. Bull.*, **62**: 328–338.

Stunkard, H. W., and C. Ruth Shaw, 1931. The effect of dilution of sea water on the activity and longevity of certain marine cercariae, with descriptions of two new species. *Biol. Bull.*, **61**: 242–271.

Stunkard, H. W., R. F. Nigrelli and C. P. Gandal, 1958. The morphology of *Renicola philippinensis* n. sp., a digenetic trematode from the pheasant-tailed jacana, *Hydrophasianus chirurgus* (Scolopi). *Zoologica, N. Y. Zool. Soc.*, **43**: 105–113.

Timon-David, J. 1933. Sur une nouvelle espèce de *Renicola*, trématode parasite du rein des laridés. Bull. Inst. Océanogr. Monaco, No. 616; pp. 1–16.

Timon-David, J., 1953. Sur une métacercaire de la sardine et ses affinités avec le groupe *Rhodometopa*. *C. R. Acad. Sci.*, **237**: 1182–1184.

Witenberg, G. G., 1929. Parasitische Würmer von *Puffinis kuhli*. *In:* Bodenheimer und Theodor, Ergebnisse der Sinai-Expedition, 1927, der Hebräischen Universität, Jerusalem; pp. 118–124. Leipzig.

Wright, C. A., 1953. Probable relationship between the *Rhodometopa* group of cercariae and the trematode genus *Renicola* Cohn. *Nature*, **171**: 1072.

Wright, C. A., 1954a. Trematodes of the genus *Renicola* from the kidneys of birds in Brazil. *Rev. Brasil. Biol.*, **14**: 61–64.

Wright, C. A., 1954b. Trematodes of the genus *Renicola* from birds in British zoos, with descriptions of two new species. *Proc. Zool. Soc. London*, **124**: 51–61.

Wright, C. A., 1956. Studies on the life-history and ecology of the trematode genus *Renicola* Cohn, 1904. *Proc. Zool. Soc. London*, **126**: 1–49.

Wright, C. A., 1957. Two kidney flukes from Sudanese birds, with a description of a new species. *J. Helminth.*, **31**: 229–238.

Yamaguti, S., 1939. Studies on the helminth fauna of Japan. Part 25. Trematodes of birds, IV. *Jap. J. Zool.*, **8**: 129–210.

Yamaguti, S., 1958. Systema Helminthum. Vol. I. The digentic trematodes of vertebrates, pp. 766–769. Interscience Publ., Inc., New York.

INDEX

ABRASIVE method for microdissection of mineralized tissues, 253.

Abdominally-produced developmental hormone in Ostrinia, 185.

Acceleration of diapause development in Ostrinia by ammonium ion, 175.

Accumulation of nitrogenous compounds in terrestrial and aquatic snail eggs, 302.

Acetate, utilization of by sea urchin embryos for lipid synthesis, 440.

Activation, role of in uptake of glucose and amino acids by marine eggs, 456.

Active transport of D-glucose by catfish intestine in vitro, 291.

Activity rhythms in bats, 14.

Adaptation of Tetrahymena to high NaCl environment, 373.

Adaptive aspects of activity rhythms in bats, 14.

Aequipecten, shell formation in, 414.

Aequorea medusae, reversible inhibition of swimming in Stomotoca by extracts of, 115.

Air-abrasive method for microdissection of mineralized tissues, 253.

Alexander, N. See S. D. Beck, 175, 185.

Allatectomy of parasitized cockroaches, 235.

Alpha-amylase activity in Crassostrea embryos, 199.

Amylase development in Crassostrea embryos, 199.

Anatomy of Homalometron, 163.

Ambystoma ear rudiment, development of in explants, 89.

Amino acids, uptake of by Asterias and Spisula eggs, 456.

Amino acids, uptake of by starfish organs in vitro, 391.

Ammonia production of snail eggs, 302.

Ammonium ion, effect of on diapause development in Ostrinia, 175.

Amphibia, erythrocyte size in, 83.

Amphibian ear rudiment, development of in explants, 89.

Amphibian embryo, regulation of liver tryptophan pyrrolase activity during, 307.

Anatomy of Renicola, 467.

Annelids, uptake of glycine by, 150.

Anomuran land crabs, water balance of, on dry atoll, 54.

Antennary glands, osmoregulatory role of, in estuarine crabs, 354.

Antisera against fertilizin, effect of on uptake of orthophosphate by sea urchin eggs, 1.

Aquatic invertebrates, uptake of organic materials by, 150.

Aquatic snails, accumulation of nitrogenous compounds in, 302.

Arbacia eggs, effect of antisera against fertilizin on orthophosphate uptake of, 1.

Arsenite, inhibition of respiration in marine dinoflagellate by, 274.

Artemia, genetics of, 333.

Aspects of activity rhythms in bats, 14.

Asterias, nutrient transport in, 33, 391.

Asterias eggs, uptake of glucose and amino acids by, 456.

Asterias gametes, method for obtaining, 8.

Asterias setting, variation in time and intensity of, 423.

Atoll, water balance of anomuran land crabs on, 54.

Auditory rudiment of amphibians, development of in explants, 89.

Autoradiographic studies of metamorphosing silkworms, 205.

Axial gradients of regeneration in planarians, 345.

Azide, inhibition of respiration in marine dinoflagellate by, 274.

BACKGROUND, role of in color change in Ocypode, 407.

Bats, rhythms of activity in, 14.

Beck, S. D., and N. Alexander. Chemically and photoperiodically induced diapause development in the European corn borer, Ostrinia, 175.

Beck, S. D., and N. Alexander. Proctodone, an insect developmental hormone, 185.

Behavior of Drosophila, in relation to light, 264.

Beta-glucosidase from Blaberus, 220.

Birgus, water balance of, on dry atoll, 54.

Blaberus, beta-glucosidase from, 220.

Black, R. E., and E. T. Pengelley. Alpha-amylase activity in embryos of Crassostrea, 199.
Blattaria, parasitized, source of juvenile hormone in, 235.
Blood cell sizes in fishes, amphibians and reptiles, 83.
Blood/urine ratios in Hemigrapsus, 354.
Bombyx, parasitized, source of juvenile hormone in, 235.
Bowen, S. T. The genetics of Artemia. IV., 333.
Bowers, B., and C. M. Williams. Physiology of insect diapause. XIII., 205.
Brine shrimp, genetics of, 333.
Brookbank, J. W. The effect of antisera against fertilizin on the uptake of orthophosphate by sea urchin eggs, 1.
Budding in Chlorohydra, as affected by Hydramoeba, 319.
Burrowing behavior of Dentalium, 28.
Busycon, shell formation in, 414.

Calcium content of mollusc shells, 414.

Carbohydrates, hydrolysis of by Blaberus enzymes, 220.
Carbon dioxide tension and sexual differentiation in Hydra, 121.
Cardioregulation in Limulus. I., 133.
Catfish, in vitro intestinal transport of D-glucose by, 291.
Cecropia silkworm, DNA synthesis during metamorphosis of, 205.
Cell sizes in fishes, amphibians and reptiles, 83.
Cercaria of Homalometron, 163.
Cercaria of Renicola, 467.
Chaet, A. B. A mechanism for obtaining mature gametes from starfish, 8.
Chemically induced diapause development in Ostrinia, 175.
Chlamys, shell formation in, 414.
Chloride, adaptation of Tetrahymena to high concentrations of, 373.
Chlorohydra, effect of Hydramoeba on reproduction of, 319.
Chromatography of cockroach enzymes, 220.
Chromatography of orthophosphate-treated sea urchin eggs, 69.
Chromatophores of Ocypode, changes in, 407.
Cipangopaludina, shell formation in, 414.
Clones, homozygous, in Rivulus, 101.
Cockroach, beta-glucosidase from, 220.
Coelenterate, effect of rhizopod infection on reproduction of, 319.
Coelenterate, sexual differentiation in, 121.
Coelomic fluid of starfish, properties of, 33.
Coenobita, water balance of, on dry atoll, 54.
Color changes in Ocypode, 407.
Corbicula, shell formation in, 414.
Corn borer, chemically and photoperiodically induced diapause in, 175.
Corn borer, developmental hormone of, 185.
Coward, S. J., R. A. Flickinger and E. Garen. The effect of nutrient media upon head frequency in regenerating planaria, 345.
Crab, color change in, 407.
Crabs, anomuran land, water balance in, on dry atoll, 54.
Crabs, estuarine, role of antennary glands in osmoregulation of, 354.
Crassostrea, shell formation in, 414.
Crassostrea embryos, development of alpha-amylase in, 199.
Cristaria, shell formation in, 414.
Crustacean, color change in, 407.
Crustacean, genetics of, 333.
Crustacean, role of antennary glands in osmotic regulation of, 354.
Cyanide inhibition of respiration in marine dinoflagellates, 274.
Cyclic activity in bats, 14.

DNA synthesis during metamorphosis of Cecropia silkworm, 205.

D_2O uptake in two brackish-water nereid polychaetes, 142.
Dark, effect of on activity rhythms of bats, 14.
Darkness, effect of on diapause development in Ostrinia, 175.
Darkness, effect of on orientation of Drosophila, 264.
Darkness, role of in color change of Ocypode, 407.
Day-length, role of in development of diapause in Ostrinia, 175.
DeCoursey, G., and P. DeCoursey. Adaptive aspects of activity rhythms in bats, 14.
Dehnel, P. A., and D. Stone. Osmoregulatory role of the antennary gland in two species of estuarine crabs, 354.
Dentalium, burrowing behavior of, 28.
Desiccation, role of in water balance of land crabs on dry atoll, 54.
Deuterium oxide uptake in two brackish-water nereid polychaetes, 142.
Developing sea urchin, synthesis of lipid by, 440.
Developing snail eggs, accumulation of nitrogenous compounds in, 302.
Development of alpha-amylase in Crassostrea embryos, 199.
Development of amphibian ear rudiment in explants, 89.

Development of diapause in Ostrinia, 175.
Development of DNA in metamorphosing silkworms, 205.
Development of Rana, regulation of liver tryptophan pyrrolase activity during, 307.
Developmental hormone in Ostrinia, 185.
D-glucose, transport of by catfish intestine in vitro, 291.
Diapause, insect, physiology of, 205.
Diapause development in Ostrinia, induced chemically and photoperiodically, 175.
Diapausing Ostrinia, effect of proctodone on, 185.
Differentiation of amphibian ear rudiment in vitro, 89.
Differentiation in vitro of planarian regenerating sections, 345.
Digenetic trematode, morphology, life-history and systematics of, 163.
Digestive glands of starfish, nutrient transport in, 391.
DINAMANI, P. Burrowing behavior of Dentalium, 28.
Dinoflagellate, marine, respiration of, 274.
Drosophila, genetic influence on phototaxis in, 264.
Dugesia, effect of nutrient media on regeneration in, 345.
DUNHAM, P. B. The adaptation of Tetrahymena to a high NaCl environment, 373.

EAR rudiment, amphibian, development of in explants, 89.
Ecdysone, during metamorphosis of silkworm, 205.
Echinoderm, nutrient transport in, 33, 391.
Echinoderm eggs, effect of antisera against fertilizin on orthophosphate uptake of, 1.
Echinoderm eggs, mechanism of phosphate transport in, 69.
Echinoderm eggs, uptake of glucose and amino acids by, 456.
Echinoderm embryos, synthesis of lipid by, 440.
Echinoderm setting, variations in time and intensity of, 423.
Ecology of starfish, 423.
Effect of antisera against fertilizin on orthophosphate uptake of sea urchin eggs, 1.
Effect of nutrient media on planarian regeneration, 345.
Egg-laying in Dentalium, 28.
Eggs, sea urchin, effect of antisera against fertilizin on orthophosphate uptake by, 1.
Eggs, sea urchin, mechanism of phosphate transport in, 69.
Eggs, sea urchin, synthesis of lipid by, 440.
Eggs, snail, accumulation of nitrogenous compounds in, 302.
Eggs of Asterias and Spisula, uptake of glucose and amino acids by, 456.
Eggs and sperm of starfish, method for obtaining, 8.
Elasmobranchs, erythrocyte size in, 83.
Electrophysiology of cardioregulation in Limulus, 133.
Electrophoresis of extrapallial fluid of molluscs, 414.
Electrophoresis of cockroach enzymes, 220.
Elliptio, shell formation in, 414.
Embryo, sea urchin, synthesis of lipid by, 440.
Embryology of amphibian ear rudiment in explants, 89.
Embryos, snail, accumulation of nitrogenous compounds in, 302.
Embryos of Crassostrea, development of alpha-amylase in, 199.
Embryos of Rana, regulation of liver tryptophan pyrrolase activity during, 307.
Enamel, sectioning of, 253.
Endocrinology of Cecropia silkworm during metamorphosis, 205.
Endocrinology of Ostrinia, 185.
Endocrinology of parasitized insects, 235.
Endogenous cycles of activity in bats, 14.
Eniwetok, water balance of land crabs on, 54.
Environment, relation of Tetrahymena to, 373.
Environmental influences on phototaxis in Drosophila, 264.
Enzyme activity in Blaberus, 220.
Enzyme activity development in oyster embryos, 199.
Enzyme activity in Rana embryos during development, 307.
Enzyme kinetics involved in uptake of glycine by Nereis, 150.
Erythrocyte measurements in fishes, amphibians and reptiles, 83.
Estuarine crabs, role of antennary glands in osmoregulation of, 354.
Excretion of aquatic and terrestrial snails, 302.
Excretion of I-131 by rainbow trout, 282.
Extrapallial fluid of molluscs, fluid protein of, 414.
Eye-color mutants in Artemia, 333.
Eyespot formation in planarian sections in vitro, 345.

FEEDING, role of in coelomic fluid constitution of starfish, 33.
Feeding, role of in parasitism of Chlorohydra by Hydramoeba, 319.
FERGUSON, J. C. Nutrient transport in starfish. I., 33; II, 391.
Fertilizability of starfish eggs obtained by injection of nerve extract, 8.

Fertilization, role of in lipid synthesis by sea urchin embryo, 440

Fertilized Asterias and Spisula eggs, uptake of glucose and amino acids by, 456.

Fertilizin, antisera against, effect of on orthophosphate uptake of sea urchin eggs, 1.

Fish, in vitro intestinal transport of D-glucose by, 291.

Fish, metabolism of I-131 by, 282.

FISHER, F. M., JR. The properties and specificity of a beta-glucosidase from Blaberus, 220.

FISHER, F. M., JR., AND R. C. SANBORN. Nosema as a source of juvenile hormone in parasitized insects, 235.

Fishes, erythrocyte size in, 83.

FLICKINGER, R. A. See S. J. COWARD, 345.

Flight activity in bats, rhythms of, 14.

Fluoroacetate, inhibition of respiration in marine dinoflagellates by, 274.

Formation of shell in molluscs, 414.

FRIBERG, U. A., P. T. LEVINE AND M. J. GLIMCHER. An air-abrasive method for the microdissection of mineralized tissues, 253.

Frog ear rudiment, development of in explants, 89.

FROMM, P. O. See J. B. HUNN, 282.

Fructose, transport of by catfish intestine in vitro, 291.

Fruit fly, genetic influence on phototaxis in, 264.

GALLERIA, allatectomized, source of juvenile hormone in, 235.

Gametes, starfish, method for obtaining, 8.

GAREN, E. See S. J. COWARD, 345.

Gastrulation, role of in lipid synthesis by sea urchin embryo, 440.

Genetic influence on phototaxis in Drosophila, 264.

Genetics of Artemia, 333.

Genotypes of self-fertilizing hermaphroditic fish, 101.

Geographical differences in Artemia populations, 333.

Germinal vesicle breakdown in Asterias and Spisula eggs, 456.

Ghost crab, morphological color change in, 407.

GLIMCHER, M. J. See U. A. FRIBERG, 253.

Glucose, transport of by catfish intestine in vitro, 291.

Glucose, uptake of by Asterias and Spisula eggs, 456.

Glucose, uptake of by isolated starfish organs, 391.

Glucosidase from Blaberus, 220.

Glycerol, utilization of by sea urchin embryo for lipid synthesis, 440.

Glycine, uptake of by brackish-water annelids, 150.

Gonad development in Hydra, in relation to carbon dioxide tension, 121.

Gonads of starfish, nutrient transport in, 391.

Gradients of regeneration in planarians, 345.

Grafts of fins in Rivulus, 101.

GREEN, J. P. Morphological color change in the Hawaiian ghost crab, Ocypode, 407.

GRIFFITHS, W. M., AND A. H. WHITELEY. A study of the mechanism of phosphate transport in sea urchin eggs by ion exchange analysis of rapidly labeled compounds, 69.

GROSS, W. J. Water balance in anomuran land crabs on a dry atoll, 54.

Growth of Ostrinia, role of proctodone in, 185.

Gut of cockroach, enzyme activity of, 220.

Gymnodinium, respiration of, 274.

HABITAT, role of in excretion patterns of snail embryos, 302.

HADLER, N. M. Genetic influence on phototaxis in Drosophila, 264.

HARRINGTON, R. W., JR. See K. D. KALLMAN, 101.

HARTMAN, F. A., AND M. A. LESSLER. Erythrocyte measurements in fishes, Amphibia and reptiles, 83.

Hawaiian ghost crab, morphological color change in, 407.

Heart action, regulation of in Limulus, 133.

Heat, role of in effect of Hydramoeba on reproduction of Chlorohydra, 319.

Heavy water, uptake of by Nereis, 142.

Hemigrapsus, role of antennary glands in osmoregulation of, 354.

Hermaphroditic teleost, homozygous clones in, 101.

Hindgut of Ostrinia, production of proctodone by, 185.

Histological sectioning of mineralized tissues, 253.

Histology of catfish intestine, 291.

Histology of metamorphosing silkworms, 205.

HOCHACHKA, P. W., AND J. M. TEAL. Respiratory metabolism in marine dinoflagellates, 274.

Homalometron, morphology, life-history and systematics of, 163.

Homozygous clones in Rivulus, 101.

Hormone, insect, 185.

Hormones, role of in DNA synthesis of metamorphosing silkworm, 205.

Horseshoe bat, activity rhythms in, 14.

Horseshoe crab, cardioregulation in, 133.

Hunn, J. R., and P. O. Fromm. Uptake, turnover and excretion of I-131 by rainbow trout (Salmo), 282.
Hyalophora, DNA synthesis during metamorphosis of, 205.
Hybridization in Artemia, 333.
Hydra, effect of Hydramoeba on reproduction of, 319.
Hydra, sexual differentiation in, in relation to carbon dioxide tension, 121.
Hydramoeba, effect of on reproduction of Chlorohydra, 319.

ICTALURUS, in vitro intestinal transport of D-glucose by, 291.
Illumination, role of in color changes of Ocypode, 407.
Immunology of sea urchin eggs, as related to orthophosphate uptake, 1.
In vitro cultivation of regenerating planarian sections, 345.
In vitro development of amphibian ear rudiment, 89.
In vitro transport of D-glucose by catfish intestine, 291.
In vitro uptake of nutrients by starfish organs, 391.
Induced diapause development in Ostrinia, 175.
Influence of genetic makeup on phototaxis in Drosophila, 264.
Inhibition of swimming in Stomotoca by mesogleal extracts of other medusae, 115.
Inhibition of unfertilized marine eggs, release of following fertilization, 456.
Inhibitor nerves, role of in cardioregulation in Limulus, 133.
Inhibitors, metabolic, effect of on distribution of orthophosphate in sea urchin eggs, 69.
Injury reaction in silkworm pupae, in relation to DNA synthesis, 205.
Insect, beta-glucosidase from, 220.
Insect diapause, physiology of, 205.
Insect hormone (proctodone), 185.
Insects, parasitized, source of juvenile hormone in, 235.
Intensity of starfish setting, variations in, 423.
Intestinal transport in catfish in vitro, 291.
Intestine, development of in oyster embryo, 199.
Intestine of cockroach, enzyme activity of, 220.
Invertebrates, uptake of organic material by, 150.
Iodoacetate, inhibition of respiration in marine dinoflagellate by, 274.
Ion exchange analysis of sea urchin eggs, 69.

JUVENILE hormone in parasitized insects, 235.

KAAN, H. W. Development of the amphibian ear rudiment in explants, 89.
Kallman, K. D., and R. W. Harrington, Jr. Evidence for the existence of homozygous clones in the self-fertilizing hermaphroditic teleost Rivulus, 101.
Kidney fluke, life-history of, 467.
Kinetics of enzyme action in Blaberus, 220.
Kobayashi, S. Studies on shell formation. X., 414.

LARVAE of Ostrinia, induction of diapause in, 175.
Larvae of Ostrinia, proctodone in, 185.
Larval oysters, alpha-amylase in, 199.
Larval Rana, liver tryptophan pyrrolase activity in, 307.
Lenhoff, H. M. Reversible inhibition of swimming Stomotoca by mesogleal extracts of some other medusae, 115.
Lessler, M. A. See F. A. Hartman, 83.
Levine, P. T. See U. A. Friberg, 253.
Life-history of Homalometron, 163.
Life-history of Renicola, 467.
Light, effect of on activity of bats, 14.
Light, effect of on diapause development in Ostrinia, 175.
Light, effect of on orientation of Drosophila, 264.
Light, role of in color changes of Ocypode, 407.
Limulus, cardioregulation in, 133.
Lipid synthesis by sea urchin embryo, 440.
Liver tryptophan pyrrolase activity in development, 307.
Long Island Sound starfish, variations in time and intensity of setting of, 423.
Loosanoff, V. L. Variations in the time and intensity of setting of the starfish, Asterias, in Long Island Sound during a 25-year period, 423.
Lytechinus eggs, effect of antisera against fertilizin on orthophosphate uptake of, 1.

MACTRA (Spisula) eggs, uptake of glucose and amino acids by, 456.
Malonate, inhibition of respiration of marine dinoflagellate by, 274.
Marine dinoflagellate, respiration of, 274.
Marisa eggs, accumulation of nitrogenous compounds in, 302
Maturation of Asterias and Spisula eggs, uptake of glucose and amino acids during, 456.
Mature starfish gametes, method for obtaining, 8.
Maze, use of in studying light-reactions of Drosophila, 264.

Measurements of erythrocyte size in fishes, Amphibia and reptiles, 83.
Mecca, C. E. See H. D. Park, 121.
Medium composition, role of in regeneration of planarian sections, 345.
Medusae, reversible inhibition of swimming in Stomotoca by extracts of, 115.
Mercenaria, shell formation in, 414.
Mercenaria shells, microdissection of, 253.
Mesogleal extracts, reversible inhibition of swimming in Stomotoca by, 115.
Metabolic inhibitors, effect of on distribution of orthophosphate in sea urchin eggs, 75.
Metabolism of glucose and amino acids by Asterias and Spisula eggs, 456.
Metabolism of lipid by sea urchin embryos, 440.
Metabolism of marine dinoflagellate, 274.
Metacercaria of Homalometron, 163.
Metacercaria of Renicola, 467.
Metal ions, effects of on Blaberus enzymes, 220.
Metamorphosis of Cecropia silkworm, DNA synthesis during, 205.
Metamorphosis of parasitized insects, 235.
Metamorphosis of Rana, liver tryptophan pyrrolase activity during, 307.
Methionine, uptake of by Asterias and Spisula eggs, 456.
Method for microdissection of mineralized tissues, 253.
Method for obtaining mature gametes from starfish, 8.
Microdissection of mineralized tissues, 253.
Mineralized tissues, microdissection of, 253.
Modiolus, shell formation in, 414.
Mohri, H. Utilization of C^{14}-labeled acetate and glycerol for lipid synthesis during the early development of the sea urchin, 440.
Mollusc, boring behavior of, 28.
Mollusc embryos, development of alpha-amylase in, 199.
Mollusc shells, microdissection of, 253.
Molluscs, shell formation in, 414.
Monroy, A., and H. Tolis. Uptake of radioactive glucose and amino acids and their utilization for incorporation into proteins during maturation and fertilization of the eggs of Asterias and Spisula, 456.
Morphological color change in Ocypode, 407.
Morphology of Homalometron, 163.
Morphology of Renicola, 467.
Mucosa of catfish intestine, transport of D-glucose by, 291.
Musacchia, X. J., S. S. Neff and D. D. Westhoff. Active transport of D-glucose by intestinal segments, in vitro, of Ictalurus, 291.
Mutant stocks of Artemia, 333.

NEANTHES, uptake of deuterium oxide by, 142.
Neanthes, uptake of glycine by, 150.
Neff, S. S. See X. J. Musacchia, 291.
Nereis, uptake of deuterium oxide by, 142.
Nereis, uptake of glycine by, 150.
Nerve extract, use of in obtaining mature starfish gametes, 8.
Neuroendocrine system of Ostrinia, 185.
Neurophysiology of cardioregulation in Limulus, 133.
Neurosecretory relations of parasitized insects, 235.
New species of Renicola, 467.
Nitrogenous compounds, accumulation of in aquatic and terrestrial snails, 302.
Nocturnal activity rhythms in bats, 14.
Nosema as source of juvenile hormone in parasitized insects, 235.
Nuclear-cytoplasmic ratios in fishes, amphibians and reptiles, 83.
Nutrient media, effect of on planarian regeneration, 345.
Nutrient transport in starfish. I., 33; II, 391.

OBTAINING mature starfish gametes, method for, 8.
Organic material, uptake of by aquatic invertebrates, 150.
Orientation of Drosophila in relation to light, 264.
Orthophosphate transport in sea urchin eggs, 69.
Orthophosphate uptake of sea urchin eggs, effect of antisera against fertilizin on, 1.
Ortmeyer, A. B. See H. D. Park, 121.
Osmoregulatory role of antennary gland in estuarine crabs, 354.
Osmotic conditions, as related to glycine uptake of Nereis, 150.
Osmotic relations of land crabs on dry atoll, 54.
Osmotic relations in Nereis, 142.
Osmotic relations of Tetrahymena, 373.
Ostrea, shell formation in, 414.
Ostrinia, chemically and photoperiodically induced diapause in, 175.
Ostrinia, developmental hormone in, 185.
Ova of Asterias and Spisula, uptake of glucose and amino acids by, 456.
Ova of sea urchin, synthesis of lipid by, 440.
Oxygen consumption of marine dinoflagellate, 274.
Oxygen consumption of metamorphosing silkworms, 205.
Oyster embryos, alpha-amylase development in, 199.

pH, effect of on Blaberus enzyme activity, 220.
Paracentrotus embryos, synthesis of lipid by, 440.
Parasitic relationship between Hydramoeba and Chlorohydra, 319.
Parasitized insects, source of juvenile hormone in, 235.
Park, H. D., C. E. Mecca and A. B. Ortmeyer. Carbon dioxide tension and sexual differentiation in Hydra, 121.
Parthenogenesis, role of in uptake of glucose and amino acids by marine eggs, 456.
Pathogenic rhizopod, effect of on reproduction of Chlorohydra, 319.
Pax, R. A., and R. C. Sanborn. Cardioregulation in Limulus. I., 133.
Pengelley, E. T. See R. E. Black, 199.
Periodism, possible role of proctodone in, 185.
Permeability relations in Nereis, 142.
Phlorizin, effect of on in vitro D-glucose transport by catfish intestine, 291.
Phosphate transport in sea urchin eggs, 69.
Phosphate uptake of sea urchin eggs, as related to action of antisera against fertilizin, 1.
Photoperiod and ammonium-ion-treated Ostrinia, action of proctodone in, 185.
Photoperiodically induced diapause development in Ostrinia, 175.
Phototaxis, genetic influence on, in Drosophila, 264.
Physiology of cardioregulatory process in Limulus, 133.
Physiology of diapause in Ostrinia, 175.
Physiology of insect diapause. XIII., 205.
Pigment changes in Ocypode, 407.
Polychaetes, deuterium oxide uptake by, 142.
Polymorphism, possible role of proctodone in, 185.
Pomacea eggs, accumulation of nitrogenous compounds in, 302.
Population differences in phototaxis of Drosophila, 264.
Population genetics of Artemia, 333.
Potassium regulation in Tetrahymena, 373.
Proctodone, an insect hormone, 185.
Properties of beta-glucosidase from Blaberus, 220.
Properties of starfish coelomic fluid, 33.
Prosobranch snail eggs, accumulation of nitrogenous compounds in, 302.
Protein, movement of in nutrition of starfish, 391.
Protein content of embryonic oysters, 199.
Proteins, synthesis of by Asterias and Spisula eggs, 456.
Proteins of extrapallial fluid in molluscs, 414.
Protozoan, adaptation of to high NaCl environment, 373.
Pyrrolase activity, liver tryptophan, during development of Rana, 307.

RADIOACTIVE glucose and amino acids, uptake of by Asterias and Spisula eggs, 456.
Radiocarbon, use of in labeling acetate and glycerol in studies of sea urchin embryo lipid synthesis, 440.
Radiocarbon, use of in studies of planarian regeneration, 345.
Radiocarbon, use of in study of starfish nutrient transport, 33, 391.
Radiocarbon, use of in study of uptake of glucose and amino acids by Asterias and Spisula eggs, 456.
Radiocarbon-labeled glycine, uptake of by Nereis, 150.
Radio-iodine, metabolism of by rainbow trout, 282.
Radiophosphorus, use of in study of phosphate transport in sea urchin eggs, 69.
Radiophosphorus, use of in studying phosphate of sea urchin eggs treated with antisera against fertilizin, 1.
Radiosodium, use of in osmotic studies with Tetrahymena, 373.
Rainbow trout, metabolism of I-131 by, 282.
Rana, regulation of liver tryptophan pyrrolase activity during development of, 307.
Rana ear rudiment, development of in explants, 89.
Rectal caeca of starfish, nutrient transport in, 391.
Redia of Homalometron, 163.
Regeneration in planarians, effect of nutrient media on, 345.
Regulation of liver tryptophan pyrrolase activity in development of Rana, 307.
Renicola, studies on, 467.
Reproduction of Chlorohydra, effect of Hydramoeba on, 319.
Reproductive isolation in Artemia, 333.
Reptiles, erythrocyte size in, 83.
Respiration of metamorphosing silkworms, 205.
Respiratory metabolism in marine dinoflagellates, 274.
Reversible inhibition of swimming in Stomotoca, 115.
Rhinolophus, activity rhythms in, 14.
Rhizopod, pathogenic, effect of on reproduction of Chlorohydra, 319.
Rhythmic production of proctodone in Ostrinia, 185.
Rhythms of activity in bats, 14.
Rivulus, homozygous clones in, 101.

SALAMANDER ear rudiment, development of in explants, 89.
Salinity, role of in osmoregulation of Hemigrapsus, 354.
Salinity in relation to glycine uptake of Nereis, 150.
Salinity relations of Tetrahymena, 373.
Salmo, metabolism of I-131 by, 282.
Salt-loss rates in Nereis, 142.
Samia, DNA synthesis during metamorphosis of, 205.
Sanborn, R. C. See F. M. Fisher, Jr., 235; R. A. Pax, 133.
Scaphopod mollusc, burrowing behavior of, 28.
Sea urchin eggs, effect of antisera against fertilizin on orthophosphate uptake by, 1.
Sea urchin eggs, mechanism of phosphate transport in, 69.
Sea urchin embryos, synthesis of lipid by, 440.
Seasonal differences in osmoregulation of Hemigrapsus, 354.
Self-fertilizing teleost, homozygous clones in, 101.
Serosa of catfish intestine, transport of D-glucose by, 291.
Serum osmotic concentration of land crabs on dry atoll, 54.
Sex-limited gene in Artemia, 333.
Sexual differentiation in Hydra, in relation to carbon dioxide tension, 121.
"Shedding substance" from starfish, use of in obtaining gametes, 8.
Shell formation, studies on, 414.
Silkworm, DNA synthesis during metamorphosis of, 205.
Size, in relation to salt-loss rate, in Nereis, 142.
Size of erythrocytes in fishes, Amphibia and reptiles, 83.
Sloan, W. C. The accumulation of nitrogenous compounds in terrestrial and aquatic eggs of prosobranch snails, 302.
Smith, R. I. D_2O uptake rate in two brackish-water nereid polychaetes, 142.
Snail eggs, accumulation of nitrogenous compounds in, 302.
Sodium, adaptation of Tetrahymena to, at high concentration, 373.
Specificity of beta-glucosidase from Blaberus, 220.
Specificity, host, of Renicola, 467.
Sperm and eggs of starfish, method for obtaining, 8.
Spiegel, M., and E. S. Spiegel. The regulation of liver tryptophan pyrrolase activity during the development of Rana, 307.
Spisula (Mactra) eggs, uptake of glucose and amino acids by, 456.
Sporocysts of Renicola, 467.
Starfish, nutrient transport in. I., 33; II, 391.
Starfish eggs, uptake of glucose and amino acids by, 456.
Starfish gametes, method for obtaining, 8.
Starfish setting, variations in time and intensity of, 423.
Starvation, role of in regeneration of planarians, 345.
Stephens, G. C. Uptake of organic material by aquatic invertebrates. III., 150.
Stiven, A. E. The effect of the pathogenic rhizopod Hydramoeba on reproduction in Chlorohydra under various levels of temperature, 319.
Stomach of starfish, nutrient transport in, 391.
Stomotoca, inhibition of swimming in, 115.
Stone, D. See P. A. Dehnel, 354.
Stress tolerance in Tetrahymena, 373.
Strongylocentrotus eggs, mechanism of phosphate transport in, 69.
Studies on genus Renicola, 467.
Studies on shell formation. X., 414.
Stunkard, H. W. The morphology, life-history and systematics of the digenetic trematode Homalometron, 163.
Stunkard, H. W. Studies on the trematode genus Renicola, 467.
Style of oysters, alpha-amylase in, 199.
Substrate, importance of in burrowing behavior of Dentalium, 28.
Substrate, role of in liver tryptophan pyrrolase activity of Rana embryos, 307.
Sulfhydryl enzyme in Blaberus gut, 220.
Swimming, inhibition of in Stomotoca, 115.
Synthesis of DNA during metamorphosis of Cecropia silkworm, 205.
Synthesis of lipid by sea urchin embryos, 456.
Systematics of genus Renicola, 467.
Systematics of genus Homalometron, 163.

TARICHA ear rudiment, development of in explants, 89.
Taxonomy of genus Renicola, 467.
Taxonomy of genus Homalometron, 163.
Teal, J. M. See P. W. Hochachka, 274.
Teeth, microdissection method for, 253.
Teleost, homozygous clones in, 101.
Teleosts, erythrocyte size in, 83.
Temperature, effect of on in vitro transport of D-glucose by catfish intestine, 291.
Temperature, role of in effect of Hydramoeba on reproduction of Chlorohydra, 319.
Temperature, role of in osmoregulation of Hemigrapsus, 354.

Temperature, role of in setting of starfish, 423.
Terrestrial snails, nitrogen compound accumulation in, *302*.
Testes of silkworms, autoradiography of, during metamorphosis, *205*.
Tetrahymena, adaptation of to high NaCl environment, *373*.
Thiocyanate, effect of on I-131 uptake by rainbow trout, *282*.
Thymidine, tritiated, use of in study of metamorphosis of silkworms, 205.
Thyroid radio-iodine, metabolism of by rainbow trout, *282*.
Tissues, mineralized, microdissection method for, *253*.
Tolis, H. See A. Monroy, 456.
Transferase activity in Blaberus, *220*.
Transplantation studies in Rivulus, 101.
Transport of D-glucose by catfish intestine in vitro, *291*.
Transport of nutrients in starfish. I., *33*; II., 391.
Transport of phosphate in sea urchin eggs, 69.
Trematode, morphology, life-history and systematics of, 163.
Trematode genus Renicola, studies on, 467.
Tribolium, source of juvenile hormone in, *235*.
Tritiated thymidine, use of in study of metamorphosis of silkworms, *205*.
Tryptophan pyrrolase activity in liver of Rana during development, *307*.
Turnover of I-131 by rainbow trout, 282.

UPTAKE of deuterium oxide by brackish-water nereids, 142.
Uptake of I-131 by rainbow trout, *282*.
Uptake of nutrients by isolated starfish organs, 391.
Uptake of organic material by aquatic invertebrates, 150.
Uptake of radioactive glucose and amino acids by Asterias and Spisula eggs, 456.
Uric acid production of snail eggs, 302.
Urinary excretion of I-131 by rainbow trout, *282*.
Urine/blood ratios in Hemigrapsus, *354*.
Urine osmotic concentrations in land crabs on dry atoll, 54.
Utilization of acetate and glycerol in lipid synthesis by sea urchin embryos, 440.

VALINE, uptake of by Spisula and Asterias eggs, 456.
Variations in time and intensity of starfish setting, *423*.
Viviparus, shell formation in, 414.

WATER balance of anomuran land crabs on a dry atoll, 54.
Water relations of Hemigrapsus, *354*.
Westhoff, D. D. See X. J. Musacchia, 291.
Whiteley, A. H. See W. M. Griffiths, 69.
Williams, C. M. See B. Bowers, 205.

XYLOSE, transport of by catfish intestine in vitro, *291*.

WH 1AZW +